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(54) Title: METHOD OF ADMINISTERING THERAPEUTIC POLYPEPTIDES, AND POLYPEPTIDES THEREFOR

(57) Abstract: The invention relates to a method suitable for administering protein therapeutic molecules orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation so as to avoid inactivation, by using VHH polypeptides derived from Camelidae antibodies. The invention further relates to the said therapeutic molecules. The invention further a method for delivering therapeutic molecules to the interior of cells. The invention furthe relates to anti-IgE therapeutic molecules.

METHOD OF ADMINISTERING THERAPEUTIC POLYPEPTIDES, AND POLYPEPTIDES THEREFOR

BACKGROUND

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, they have one important drawback: these are complex, large molecules and therefore relatively unstable, and they are sensitive to breakdown by proteases. Because the degradation they undergo during passage through, for instance, the gastrointestinal tract, administration of conventional antibodies and their derived fragments or single-chain formats (e.g. scFv's) is not very effective. This means that conventional antibody drugs cannot be administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation because they are not resistant to the low pH at these sites, the action of proteases at these sites and in the blood and/or because of their large size. They have to be administered by injection (intravenously, subcutaneously, etc.) to overcome some of these problems. Administration by injection is therefore the most frequently used method of administration although the method has many disadvantages, for example: (a) poor tolerance by patients, especially when treating chronic disorder; (b) a consequent risk of poor compliance with the dosage when the drug is not a 'life saver'; (c) difficulty of carrying out self-administration by the patient; (d) possible non-availability of suitable surroundings for carrying out the procedure in an aseptic manner; (e) requires specialist training in order to use a hypodermic syringe or needle correctly and safely. A method for the delivery of therapeutic polypeptides which avoids the need for injection has not only cost/time savings, but would also be more convenient and more comfortable for the subject.

In most animal cells, a specialised pathway is present for uptake of specific macromolecules from the extracellular fluid. The macromolecules that bind to specific cell-surface receptors are internalized, a process called receptor-mediated endocytosis. Receptor internalization is based on the principle of regulation of signal transduction by a process called sequestration, whereby bound agonistic (i.e. receptor activation) ligands are recovered from the cell surface in complex with the receptor. For many applications it is necessary to deliver effector molecules across the cell membrane and into the cytosol. This can be achieved by taking advantage of such internalizing receptors. Antibodies have been described that internalize

upon binding to internalizing receptors. However, they have important drawbacks: these antibodies are complex, large molecules and therefore relatively unstable, and they are sensitive to breakdown by proteases. Moreover, the domains of such antibodies are held together by disulphide bonds that dissociate in the reducing environment of the cytoplasm leading to a substantial loss of binding activity. Therefore, they cannot be used to target intracellular proteins.

Another process that relies on internalisation is the efficient induction of an immune response. In particular, a T-cell response depends heavily on efficient presentation of certain epitopes to the T cells by antigen presenting cells (APCs). In the case of a protein antigen this means that the APC has to take up the protein, internally process it (this is cleaving it) and express certain peptide fragments on its surface in association with MHC (major histocompatibility complex) or HLA molecules. One major and critical event in this process is the efficient uptake of the protein antigen by its APC. Techniques which can enhance antigen uptake by APCs enables an immune response to be elicited against antigens which naturally elicit a weak or no immune response. Therefore, a technique which can boost an immune response against antigenic antigens, naturally weak or non-immunogenic antigens has important implications for vaccination programs.

IgE plays a major role in allergic disease by causing the release of histamine and other inflammatory mediatord from mast cells. A mainstay of treatment of allergic disease, including asthma, is allergen avoidance and treatment of symptoms. Presently, the most effective treatments of allergic diseases are directed towards a regulation of the inflammatory process with corticosteroids. A more direct approach without the negative effects of corticosteroids consists in regulating the allergic process at the level of the initiatior of the allergic inflammation, IgE, via an anti-IgE.

The concept of using anti-IgE antibodies as a treatment for allergy has been widely disclosed in the scientific literature. A few representative examples are as follows. Baniyash and Eshhar (European Journal of Immunology 14:799-807 (1984)) demonstrated that an anti-IgE monoclonal antibody could specifically block passive cutaneous anaphylaxis reaction when injected intradermally before challenging with the antigen; U.S. 4,714,759 discloses a product and process for treating allergy, using an antibody specific for IgE; and Rup and Kahn (International Archives Allergy and Applied Immunology, 89:387-393 (1989) discuss the

prevention of the development of allergic responses with monoclonal antibodies which block mast cell-IgE sensitization.

Anti-IgE antibodies which block the binding of IgE to its receptor on basophils and which fail to bind to IgE bound to the receptor, thereby avoiding histamine release are disclosed, for example, by Rup and Kahn (supra), by Baniyash et al. (Molecular Immunology 25:705-711, 1988), and by Hook et al. (Federation of American Societies for Experimental Biology, 71st Annual Meeting, Abstract #6008, 1987).

Antagonists of IgE in the form of receptors, anti-IgE antibodies, binding factors, or fragments thereof have been disclosed in the art. For example, U.S. 4,962,035 discloses DNA encoding the alpha-subunit of the mast cell IgE receptor or an IgE binding fragment thereof. Hook et al. (Federation Proceedings Vol. 40, No. 3, Abstract #4177) disclose monoclonal antibodies, of which one type is anti-idiotypic, a second type binds to common IgE determinants, and a third type is directed towards determinants hidden when IgE is on the basophil surface.

U.S. 4,940,782 discloses monoclonal antibodies which react with free IgE and thereby inhibit IgE binding to mast cells, and react with IgE when it is bound to the B-cell FcE receptor, but do not bind with IgE when it is bound to the mast cell FcE receptor, nor block the binding of IgE to the B-cell receptor.

U.S. 4,946,788 discloses a purified IgE binding factor and fragments thereof, and monoclonal antibodies which react with IgE binding factor and lymphocyte cellular receptors for IgE, and derivatives thereof.

U.S. 5,091,313 discloses antigenic epitopes associated with the extracellular segment of the domain which anchors immunoglobulins to the B cell membrane. The epitopes recognized are present on IgE-bearing B cells but not basophils or in the secreted, soluble form of IgE. U.S. 5,252,467 discloses a method for producing antibodies specific for such antigenic epitopes. U.S. 5,231,026 discloses DNA encoding murine-human antibodies specific for such antigenic epitopes.

U.S. 4,714,759 discloses an immunotoxin in the form of an antibody or an antibody fragment coupled to a toxin to treat allergy.

Presta et al. (J. Immunol. 151:2623-2632 (1993)) disclose a humanized anti-IgE antibody that prevents the binding of free IgE to FceRI but does not bind to Fc∈RI-bound IgE. Copending WO93/04173 discloses polypeptides which bind differentially to the high- and low-affinity IgE receptors.

U.S. 5,428,133 discloses anti-IgE antibodies as a therapy for allergy, especially antibodies which bind to IgE on B cells, but not IgE on basophils. This publication mentions the possibility of treating asthma with such antibodies. U.S. 5,422,258 discloses a method for making such antibodies.
EP0841946 discloses methods for treating allergic asthma using IgE antagonists.

AIMS OF THE INVENTION

The aim of the invention is to provide a method of administering protein therapeutic molecules orally, sublingually, topically, nasally, vaginally, rectally, intraveneously, subcutaneously or by inhalation which overcomes the problems of the prior art. It is a further aim to provide said therapeutic molecules.

Another aim of the invention is to provide a method for delivering therapeutic substances to the interior of cells via internalizing receptors without receptor activation.

It is further aim of the invention to provide a therapeutic agent for the treatment of allergies.

It is a further aim of the invention to provide therapeutic nanobodies.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against IgE.

Another embodiment of the present invention is a polypeptide construct as described above wherein at least one single domain antibody is a Camelidae VHH.

Another embodiment of the present invention is a polypeptide construct as described above wherein at least one single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 11.

Another embodiment of the present invention is a polypeptide construct as described above, wherein the number of anti-IgE single domain antibodies is at least two.

Another embodiment of the present invention is a polypeptide construct as described above, wherein at least one single domain antibody is a humanized Camelidae VHH.

Another embodiment of the present invention is a polypeptide construct as described above, wherein a single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

Another embodiment of the present invention is a polypeptide construct as described above, wherein the polypeptide construct is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length polypeptide construct.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as described above.

Another embodiment of the present invention is a polypeptide construct as described above for treating and/or preventing and/or alleviating disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of a polypeptide construct as described above for the preparation of a medicament for treating and/or preventing and/or alleviating disorders relating to inflammatory reactions.

Another embodiment of the present invention is a method for delivering an anti-target compound to a subject for the treatment of a disorder without being inactivated by administering thereto a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a method as described above wherein said target is located in the gut system, and said a polypeptide construct is delivered orally.

Another embodiment of the present invention is a method as described above wherein said target is located in vaginal and/or rectal tract, and said a polypeptide construct is delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is a method as described above wherein said target is located in nose, upper respiratory tract and/or lung; and said a polypeptide construct is delivered to nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is a method as described above wherein said target is located in intestinal mucosa, and said a polypeptide construct is delivered orally.

Another embodiment of the present invention is a method as described above wherein said target is located in the tissues beneath the tongue, and said a polypeptide construct is delivered to the tissues beneath the tongue.

Another embodiment of the present invention is a method as described above wherein said target is located in the skin, and said a polypeptide construct is delivered topically.

Another embodiment of the present invention is a method as described above wherein said target is in, or accessible via the blood, and said a polypeptide construct is delivered orally, to the vaginal and/or rectal tract, nasally, by inhalation though the mouth or nose, to the tissues beneath the tongue, or topically.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target, for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of the intestinal mucosa without being inactivated

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or

alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of the nose, upper respiratory tract and/or lung without being inactivated

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of virginal and/or rectal tract without being inactivated

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by a therapeutic compound that is able pass through the tissues beneath the tongue without being inactivated

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by a therapeutic compound that is able pass through the skin without being inactivated

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is TNF-alpha and the disorder is inflammation.

Another embodiment of the present invention is a method or polypeptide as described above, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 12 to 14.

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is CEA and the disorder colon cancer.

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is EGFR and the disorder is any of head, neck, lung and colon cancer.

Another embodiment of the present invention is a method or polypeptide construct as described above, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 23 to 44

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of Helicobacter pylori and the disorder is any of indigestion, gastritis..

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of Mycobacterium tuberculosis and the disorder is tuberculosis.

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of influenza virus and the disorder is flu.

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of IgE and the disorder is allergic response.

Another embodiment of the present invention is a method or polypeptide construct as described above, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 11

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of MMP and the disorder is cancer.

Another embodiment of the present invention is a method or polypeptide construct as described above, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 15 to 22

Another embodiment of the present invention is a method as described above or polypeptide construct as described above, wherein said target is antigen of IFN-gamma and the disorder is any of cancer, transplant rejection, auto immune disorder.

Another embodiment of the present invention is a method or polypeptide construct as described above, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 45 to 70

Another embodiment of the present invention is a method as described above or polypeptide construct as described above wherein said target is any of antigen of Helicobacter pylori, antigen of Mycobacterium tuberculosis, antigen of influenza virus.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, and at least one single domain antibody directed against a therapeutic target.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, and at least one therapeutic polypeptide or agent.

Another embodiment of the present invention is a polypeptide construct as described above wherein said internalising cellular receptor is Epidermal Growth Factor receptor.

Another embodiment of the present invention is a polypeptide as described above wherein a single domain antibody directed against an internalising cellular receptor corresponds to a sequence represented by SEQ ID NO: 23 to 44.

Another embodiment of the present invention is a polypeptide construct as described above wherein said internalising cellular receptor is any of LDL receptor, FGF2r, ErbB2r, transferring receptor, PDGr, VEGr, or PsmAr.

Another embodiment of the present invention is a polypeptide construct as described above wherein a single domain antibody directed against a therapeutic target, is directed against PDK1.

Another embodiment of the present invention is a polypeptide construct as described above use in treating cancer

Another embodiment of the present invention is a polypeptide construct as described above wherein a single domain antibody directed against a therapeutic target is directed against any of GSK1, Bad, caspase and Forkhead.

Another embodiment of the present invention is a polypeptide construct as described above use in treating cancer.

Another embodiment of the present invention is a method for delivering an anti-target therapeutic compound to the interior of a cell comprising administering to a subject a polypeptide construct as described above.

Another embodiment of the present invention is a method for delivering an anti-target therapeutic compound to the interior of a cell without being inactivated comprising administering to a subject a polypeptide construct as described above.

Another embodiment of the present invention is a method as described above wherein said cell is located in the gut system, and said a polypeptide construct is delivered orally.

Another embodiment of the present invention is a method as described above wherein said cell is located in vaginal and/or rectal tract, and said a polypeptide construct is delivered to the vaginal and/or rectal tract.

Another embodiment of the present invention is a method as described above wherein said cell is located in nose, upper respiratory tract and/or lung, and said a polypeptide construct is delivered to nose, upper respiratory tract and/or lung.

Another embodiment of the present invention is a method as described above wherein said cell is located in intestinal mucosa, and said a polypeptide construct is delivered orally.

Another embodiment of the present invention is a method as described above wherein said cell is located in the tissues beneath the tongue, and said a polypeptide construct is delivered to the tissues beneath the tongue.

Another embodiment of the present invention is a method as described above wherein said cell is located in the skin, and said a polypeptide construct is delivered topically.

Another embodiment of the present invention is a method as described above wherein said cell is in, or accessible via the blood, and said a polypeptide construct is delivered orally, to the vaginal and/or rectal tract, nasally, by inhalation though the mouth or nose, to the tissues beneath the tongue, or topically.

Another embodiment of the present invention is a polypeptide construct as described above, or a method as described above, wherein the single domain antibodies are humanized Camelidae VHHs.

Another embodiment of the present invention is a polypeptide construct as described above, or a method as described above, wherein said single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

Another embodiment of the present invention is a polypeptide construct as described above or a method as described above, wherein the polypeptide construct is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length polypeptide construct.

Another embodiment of the present invention is a polypeptide construct as described above or a method as described above wherein said single domain antibodies are Camelidae VHHs.

Another embodiment of the present invention is a nucleic acid capable of encoding a polypeptide construct as described above.

Another embodiment of the present invention is a composition comprising a polypeptide construct as defined above, together with a pharmaceutical carrier.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a polypeptide construct comprising one or more single domain antibodies directed to one or more target molecule(s), each in a suitable dosage form either directly or as part of a composition containing an ingredient which facilitates delivery.

The invention further relates to polypeptide constructs comprising one or more single domain antibodies, for administration to a subject by non-invasive methods, such as orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Such non-invasive routes of delivery unexpectly provide an effective means to conveniently deliver therapeutic compounds

The present invention also relates to constructs comprising one or more single domain antibodies, for administration to a subject by normal invasive methods such as intravenously and subcutaneously.

The invention further relates to a method for delivering therapeutic peptides comprises the steps of administering a polypeptide construct comprising one or more single domain antibodies orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation to a subject.

The invention further relates to polypeptide constructs comprising anti-IgE single domain antibodies.

Single domain antibodies are antibodies whose complementary determining regions are part of a single domain polypetide. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived from antibodies. Single domain antibodies may be any of the art, or any future single domain antibodies. Single domain antibodies may be derived from any species including, but not limited to mouse, human, camel, llama, goat, rabbit, bovine. According to one aspect of the invention, a single domain antibody as used herein is a naturally occurring single domain antibody known as heavy chain antibody devoid of light chains. Such single domain antibodies are disclosed in WO 9404678 for example. For clarity reasons, this variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a VHH or nanobody to distinguish it from the conventional VH of four chain immunoglobulins. Such a VHH molecule can be derived from antibodies raised in *Camelidae* species, for example in camel, llama, dromedary, alpaca and guanaco. Other species besides *Camelidae* may produce heavy chain antibodies naturally devoid of light chain; such VHHs are within the scope of the invention.

VHHs, according to the present invention, and as known to the skilled addressee are heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from *Camelidae* as described in WO 94/04678 (and referred to hereinafter as VHH domains or nanobodies). VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies. Furthermore, *in vitro* expression of VHHs produces high yield, properly folded functional VHHs. In addition, antibodies generated in *Camelids* will recognize epitopes other than those recognised by antibodies generated *in vitro* through the use of antibody libraries or via immunisation of mammals other than *Camelids* (WO 9749805). As such, anti-albumin VHH's may interact in a more efficient way with serum albumin which is known to be a carrier protein. As a carrier protein some of the epitopes of serum albumin may be inaccessible by bound proteins, peptides and small chemical compounds. Since VHH's are known to bind into 'unusual' or non-conventional epitopes such as cavities (WO 97/49805), the affinity of such VHH's to circulating albumin may be increased.

The present invention further relates to a polypeptide construct, wherein a single domain antibody is a VHH directed against a target, wherein the VHH belongs to a class having human-like sequences. The class is characterised in that the VHHs carry an amino acid from the group consisting of glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, methionine, serine, threonine, asparagine, or glutamine at position 45, such as, for example, L45 according to the Kabat numbering. A VHH sequence represented by SEQ ID NO: 15 which binds to MMP-12, belongs to this human-like class of VHH polypeptides. As such, peptides belonging to this class show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanisation.

Another human-like class of *Camelidae* single domain antibodies represented by sequences 68 which binds to IFN gamma , have been described in WO03035694 and contain the hydrophobic FR2 residues typically found in conventional antibodies of human origin or from other species, but compensating this loss in hydrophilicity by the charged arginine residue on position 103 that substitutes the conserved tryptophan residue present in VH from conventional antibodies. As such, peptides belonging to these two classes show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to a human directly without expectation of an unwanted immune response therefrom, and without the burden of further humanisation.

Any of the VHHs as used by the invention may be of the traditional class or of the classes of human-like *Camelidae* antibodies. Said antibodies may be directed against whole target or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include the full length *Camelidae* antibodies, namely Fc and VHH domains, chimeric versions of heavy chain *Camelidae* antibodies with a human Fc domain.

Targets of the invention are any which are of pharmaceutical interest. Examples are provided here of several targets, and are not intended to limit the invention thereto. Examples of targets include, TNF-alpha, IgE, IFN-gamma, MMP-12, EGFR, CEA, H. pylori, TB, influenza. A single domain antibody directed against a target means a single domain antibody that is capable of binding to said target with an affinity of better than 10^{-6}M.

Targets may also be fragments of said targets. Thus a target is also a fragment of said target, capable of eliciting an immune response. A target is also a fragment of said target, capable of binding to a single domain antibody raised against the full length target.

A fragment as used herein refers to less than 100% of the sequence (e.g., 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% etc.), but comprising 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids. A fragment is of sufficient length such that the interaction of interest is maintained with affinity of 1 x 10^{-6} M or better.

A fragment as used herein also refers to optional insertions, deletions and substitutions of one or more amino acids which do not substantially alter the ability of the target to bind to a single domain antibody raised against the wild-type target. The number of amino acid insertions deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

One embodiment of the present invention is a polypeptide construct as disclosed herein, wherein the number of single domain antibodies directed to a target is two or more. Such multivalent polypeptide constructs have the advantage of unusually high functional affinity for the target, displaying much higher than expected inhibitory properties compared to their monovalent counterparts.

Multivalent polypeptide constructs have functional affinities that are several orders of magnitude higher than polypeptide constructs which are monovalent. The inventors have found that the functional affinities of these multivalent polypeptides are much higher than those reported in the prior art for bivalent and multivalent antibodies. Surprisingly, the multivalent polypeptide constructs of the present invention linked to each other directly or via a short linker sequence show the high functional affinities expected theoretically with multivalent conventional four-chain antibodies.

The inventors have found that such large increased functional activities can be detected preferably with antigens composed of multidomain and multimeric proteins, either in straight binding assays or in functional assays, *e.g.* animal model of chronic colitis.

A multivalent anti-target polypeptide as used herein refers to a polypeptide comprising two or more anti-target polypeptides which have been covalently linked. The anti-target polypeptides may be identical in sequence or may be different in sequence, but are directed against the same target or antigen. Depending on the number of anti-target polypeptides linked, a multivalent anti-target polypeptide may be bivalent (2 anti-target polypeptides), trivalent (3 anti-target polypeptides), tetravalent (4 anti-target polypeptides) or have a higher valency molecules.

An example of a multivalent polypeptide construct of the invention, comprising more than one anti-TNF-alpha VHHs is described in Example 7.

The single domain antibodies may be joined to form any of the polypeptide constructs disclosed herein comprising more than one single domain antibody using methods known in the art or any future method. They may be joined non-covalently (e.g. using streptavidin/biotin combination, antibody/tag combination) or covalently. They may be fused by chemical cross-linking by reacting amino acid residues with an organic derivatising agent such as described by Blattler *et al*, Biochemistry **24**,1517-1524; EP294703. Alternatively, the single domain antibody may be fused genetically at the DNA level *i.e.* a polynucleotide construct formed which encodes the complete polypeptide construct comprising one or more anti-target single domain antibodies. A method for producing bivalent or multivalent VHH polypeptide constructs is disclosed in PCT patent application WO 96/34103. One way of joining VHH antibodies is via the genetic route by linking a VHH antibody coding sequences either directly or via a peptide linker. For example, the C-terminal end of the VHH antibody may be linked to the N-terminal end of the next single domain antibody.

This linking mode can be extended in order to link additional single domain antibodies for the construction and production of tri-, tetra-, etc. functional constructs.

According to one aspect of the present invention, the single domain antibodies are linked to each other via a peptide linker sequence. Such linker sequence may be a naturally occurring sequence or a non-naturally occurring sequence. The linker sequence is expected to be non-immunogenic in the subject to which the multivalent anti-target polypeptide is administered. The linker sequence may provide sufficient flexibility to the multivalent anti-target polypeptide, at the same time being resistant to proteolytic degradation. A non-limiting example of a linker sequences is one that can be derived from the hinge region of VHHs described in WO 96/34103.

The polypeptide constructs disclosed herein may be made by the skilled artisan according to methods known in the art or any future method. For example, VHHs may be obtained using methods known in the art such as by immunising a camel and obtaining hybridomas therefrom, or by cloning a library of single domain antibodies using molecular biology techniques known in the art and subsequent selection by using phage display.

According to an aspect of the invention a polypeptide construct may be a homologous sequence of a full-length polypeptide construct. According to another aspect of the invention, a polypeptide construct may be a functional portion of a full-length polypeptide construct. According to another aspect of the invention, a polypeptide construct may be a homologous sequence of a full length polypeptide construct. According to another aspect of the invention, a polypeptide construct may be a functional portion of a homologous sequence of a full length polypeptide construct. According to an aspect of the invention a polypeptide construct may comprise a sequence of a polypeptide construct.

According to an aspect of the invention a single domain antibody used to form a polypeptide construct may be a complete single domain antibody (*e.g.* a VHH) or a homologous sequence thereof. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a functional portion of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a homologous sequence of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the polypeptide construct may be a functional portion of a homologous sequence of a complete single domain antibody.

As used herein, a homologous sequence of the present invention may comprise additions, deletions or substitutions of one or more amino acids, which do not substantially alter the functional characteristics of the polypeptides of the invention. The number of amino acid deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence according to the present invention may be a sequence of an anti-target polypeptide modified by the addition, deletion or substitution of amino acids, said modification not substantially altering the functional characteristics compared with the unmodified polypeptide.

A homologous sequence of the present invention may be a polypeptide which has been humanised. The humanisation of antibodies of the new class of VHHs would further reduce the possibility of unwanted immunological reaction in a human individual upon administration.

A homologous sequence according to the present invention may be a sequence which exists in other *Camelidae* species such as, for example, camel, llama, dromedary, alpaca, guanaco etc.

Where homologous sequence indicates sequence identity, it means a sequence which presents a high sequence identity (more than 70%, 75%, 80%, 85%, 90%, 95% or 98% sequence identity) with the parent sequence and is preferably characterised by similar properties of the parent sequence, namely affinity, said identity calculated using known methods.

Alternatively, a homologous sequence may also be any amino acid sequence resulting from allowed substitutions at any number of positions of the parent sequence according to the formula below:

Ser substituted by Ser, Thr, Gly, and Asn;

Arg substituted by one of Arg, His, Gln, Lys, and Glu;

Leu substituted by one of Leu, Ile, Phe, Tyr, Met, and Val;

Pro substituted by one of Pro, Gly, Ala, and Thr;
Thr substituted by one of Thr, Pro, Ser, Ala, Gly, His, and Gln;
Ala substituted by one of Ala, Gly, Thr, and Pro;
Val substituted by one of Val, Met, Tyr, Phe, Ile, and Leu;
Gly substituted by one of Gly, Ala, Thr, Pro, and Ser;
Ile substituted by one of Ile, Met, Tyr, Phe, Val, and Leu;
Phe substituted by one of Phe, Trp, Met, Tyr, Ile, Val, and Leu;
Tyr substituted by one of Tyr, Trp, Met, Phe, Ile, Val, and Leu;
His substituted by one of His, Glu, Lys, Gln, Thr, and Arg;
Gln substituted by one of Gln, Glu, Lys, Asn, His, Thr, and Arg;
Asn substituted by one of Asn, Glu, Asp, Gln, and Ser;
Lys substituted by one of Lys, Glu, Gln, His, and Arg;
Asp substituted by one of Asp, Glu, and Asn;
Glu substituted by one of Glu, Asp, Lys, Asn, Gln, His, and Arg;
Met substituted by one of Met, Phe, Ile, Val, Leu, and Tyr.

A homologous nucleotide sequence according to the present invention may refer to nucleotide sequences of more than 50, 100, 200, 300, 400, 500, 600, 800 or 1000 nucleotides able to hybridize to the reverse-complement of the nucleotide sequence capable of encoding the patent sequence, under stringent hybridisation conditions (such as the ones described by Sambrook *et al.*, Molecular Cloning, Laboratory Manuel, Cold Spring, Harbor Laboratory press, New York).

As used herein, a functional portion refers to a sequence of a single domain antibody that is of sufficient size such that the interaction of interest is maintained with affinity of 1 x 10^{-6} M or better.

Alternatively, a functional portion comprises a partial deletion of the complete amino acid sequence and still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with its target.

As used herein, a functional portion refers to less than 100% of the complete sequence (*e.g.*, 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 1% etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

ANTI-IgE SINGLE DOMAIN ANTIBODIES

One aspect of the present invention relates to therapeutic compounds which are suitable for alleviating the symptoms, for the treatment and prevention of allergies. Said therapeutic compounds interact with IgE, and modulate the cascade of immunological responses that is responsible for an allergic response.

Another aspect of the present invention relates to the use of anti-IgE single domain antibodies (e.g. VHHs)in the preparation of topical ophthalmic compositions for the treatment of an ocular allergic disorder (Example 2). Given the ease of production and the low cost using bacterial or yeast expression systems for VHHs, for example, compared to production of conventional antibodies in mammalian cells, the economics of preparing such compositions using VHHs of the invention are much more favourable then for conventional antibodies.

Ocular penetration and consequently ocular efficacy is highly unexpected with conventional antibodies and derived fragments given their large size. The polypeptide constructs of the invention however are expected to be highly efficient given their high potency, stability combined with a low molecular weigth. Therefore, applications for such indications other than topical can be envisaged with polypeptide constructs of the invention.

One embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies directed against IgE.

Another embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies directed against IgE, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 11. Said sequences are derived from *Camelidae* VHHs.

The present invention also relates to the finding that a polypeptide construct comprising one or more single domain antibodies directed against IgE and further comprising one or more single domain antibodies directed against one or more serum proteins of a subject, surprisingly has significantly prolonged half-life in the circulation of said subject compared with the half-life of the anti-IgE single domain antibody when not part of said construct. Furthermore, such polypeptide constructs were found to exhibit the same favourable properties of VHHs such as high stability remaining intact in mice, extreme pH resistance, high temperature stability and high target affinity.

Another embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies directed against IgE further comprising one or more single domain antibodies directed against one or more serum proteins.

The serum protein may be any suitable protein found in the serum of subject, or fragment thereof. In one aspect of the invention, the serum protein is serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, or fibrinogen. Depending on the intended use such as the required half-life for effective treatment and/or compartimentalisation of the target antigen, the VHH-partner can be directed to one of the above serum proteins.

One aspect of the invention, is a polypeptide construct comprising one or more single domain antibodies directed against IgE, further comprising an anti-serum albumin single domain antibody corresponding to a sequence represented by any of SEQ ID NO: 71 to 84.

DELIVERY OF POLYPEPTIDE CONSTRUCTS

The aspect of the invention relating to the delivery of polypeptide constructs of the invention is not limited to a polypeptide construct comprising anti-IgE single domain antibodies disclosed herein, but, as shown below, is applicable to any target. The polypeptide constructs may comprise single domain antibodies directed against more than one target, optionally with the variations described above.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or

alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

As known by persons skilled in the art, once in possession of said polypeptide construct, formulation technology may be applied to release a maximum amount of VHHs in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disorder whose targets that are located in the gut system.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder susceptible to modulation by a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies specific for antigen related to the disorder.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the gut system without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound to the vaginal and/or rectal tract.

In a non-limiting example, a formulation according to the invention comprises a polypeptide construct as disclosed herein comprising one or more VHHs directed against one or more targets in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time (such formulations are described in EP 707473, EP 684814, US 5629001).

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject a polypeptide construct comprising one or more single domain antibodies specific for antigen related to the disorder.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound to the vaginal and/or rectal tract without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the vaginal and/or rectal tract without being inactivated, by administering to the vaginal and/or rectal tract of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by administering to the vaginal and/or rectal tract of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target comprising at least one single domain antibody directed against a target, for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound to the nose, upper respiratory tract and/or lung.

In a non-limiting example, a formulation according to the invention, comprises a polypeptide construct as disclosed herein directed against one or more targets in the form of a nasal spray (e.g. an aerosol) or inhaler. Since the construct is small, it can reach its target much more effectively than therapeutic IgG molecules.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound to the upper respiratory tract and lung, by administering to a subject a polypeptide construct as disclosed herein wherein one or more single domain antibodies are specific for an antigen related to the disorder, by inhalation through the mouth or nose.

Another aspect of the invention is a dispersible VHH composition, in particular dry powder dispersible VHH compositions, such as those described in US 6514496. These dry powder compositions comprise a plurality of discrete dry particles with an average particle size in the range of 0.4-10 μm. Such powders are capable of being readily dispersed in an inhalation device. VHH's are particularly suited for such composition as lyophilized material can be readily dissolved (in the lung subsequent to being inhaled) due to its high solubilisation capacity (Muyldermans, S., Reviews in Molecular Biotechnology, 74, 277-303, (2001)). Alternatively, such lyophilized VHH formulations can be reconstituted with a diluent to generate a stable reconstituted formulation suitable for subcutaneous administration. For example, anti-IgE antibody formulations (Example 1; US 6267958, EP 841946) have been prepared which are usefull for treating allergic asthma.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound to to the nose, upper respiratory tract and/or lung without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the nose, upper respiratory tract and lung, by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the nose, upper respiratory tract and/or lung without being inactivated, by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is increased. Because of their small size, a polypeptide construct as disclosed herein can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is increased, by orally administering to a subject a polypeptide construct as disclosed herein comprising one or more single domain antibodiesspecific for an antigen related to the disorder.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, VHH is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a second VHH which is fused to the therapeutic VHH. Such fusion constructs made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound to

the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the intestinal mucosa without being inactivated, by administering orally to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by administering orally to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the tissues beneath the tongue effectively. A formulation of said polypeptide construct as disclosed herein, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound that is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject a VHH specific for an antigen related to the disorder.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able to pass through the tissues beneath the tongue.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the tissues beneath the tongue without being inactivated, by administering orally to a subject

a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inecativated, by administering orally to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the skin effectively. A formulation of said polypeptide construct, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound that is able pass through the skin effectively, by topically administering to a subject a polypeptide construct as disclosed herein comprising one or more single domain antibodies specific for an antigen related to the disorder.

Another aspect of the invention is the use of a polypeptide construct as disclosed herein as a topical ophthalmic composition for the treatment of ocular disorder, such as allergic disorders, which method comprises the topical administration of an ophthalmic composition comprising polypeptide construct as disclosed herein, said construct comprising one or more anti-IgE VHH (Example 1, Example 2).

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or aleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the skin effectively.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the skin without being inactivated, by administering topically to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject, by administering topically to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

A non-limiting example of a therapeutic target against which a polypeptide construct of the invention may be used is TNF, which is involved in inflammatory processes. The blocking of TNF action can have an anti-inflammatory effect, which is highly desirable in certain disease states such as, for example, Crohn's disease. Current therapy consists of intravenous administration of anti-TNF antibodies. Our Examples (Example 4) demonstrate VHHs according to the invention which bind TNF and moreover, block its binding to the TNF receptor. Oral delivery of these anti-TNF polypeptide constructs results in the delivery of such molecules in an active form in the colon at sites that are affected by the disorder. These sites are highly inflamed and contain TNF-producing cells. These anti-TNF polypeptide constructs can neutralise the TNF locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments (US 6190662, WO 0023471). Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a TNF neutralizing antibody fragment, inflammatory bowel disorder could be treated. Another aspect of the invention is a polypeptide construct comprising at least one single domain antibody specific for TNF-alpha for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said polypeptide construct is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to inflammatory processes, comprising administering to a subject a polypeptide construct comprising at least one single domain antibody directed against for example TNF-alpha orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation.

According to one aspect of the invention, a polypeptide construct of the invention comprises at least one single domain antibody directed against TNF-alpha, said single domain antibody corresponding to a sequence represented by any of SEQ ID NOs: 12 to 14. Said sequences are anti-TNF-alpha *Camelidae* VHHs.

Further non-limiting examples of therapeutic targets against which a polypeptide construct of the invention may be used are certain colon cancer specific antigens, such as, for example, CEA or EGF receptors. In one aspect of the invention, therapeutic VHHs against colon cancer antigens are linked to or provided with one more tumor destroying reagents such as for example, a chemical compound or a radioactive compound.

As stated above a colon cancer specific antigen according to the invention is epidermal growth factor receptor (EGFR) which is an essential mediator of cell division in mammalian cells and is a recognised cellular oncogene. After the binding of EGF to its receptor (EGFR), a signaling cascade is initiated resulting in cell development. The EGFR is also involved in human tumorigenesis as it is overexpressed on cells associated with epithelial malignancies located in sites such as the head, neck, lung, colon. Another aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against EGFR for use in the treatment, prevention and/or alleviation of disorders relating to EGFR-mediated cancer, wherein said VHH is administered orally, sublingually, topically, nasally, intravenously, subcutaneously, vaginally, rectally or by inhalation (Examples 25 to 31). Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to EGFR-mediated cancer, comprising administering to a subject a polypeptide construct comprising at least one single domain antibody directed against EGFR orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation.

According to one aspect of the invention, a polypeptide construct of the invention comprises at least one single domain antibody directed against EGFR, said single domain antibody corresponding to a sequence represented by any of SEQ ID NOs: 23 to 44. Said sequences are anti-EGRF *Camelidae* VHHs.

As stated above another colon cancer specific antigen according to the invention is carcinoembryonic antigen (CEA), a recognized tumor marker. Another aspect of the invention is a polypeptide construct comprising one or more single domain antibodies specific for CEA for use in the treatment, prevention and/or alleviation of disorders relating to CEA-mediated cancer, wherein said polypeptide is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to CEA-mediated cancer, comprising administering to a subject a polypeptide construct comprising at least one single domain antibody directed against CEA, orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. A few VHHs specific for this glycoprotein have been isolated by selection on solid-phase coated with CEA out of a dedicated library obtained after immunization of a dromedary. By using FACS analysis it appeared that only two fragments recognized the cell-bound antigen. One of the VHHs, that recognised the native structure, has been used to construct a fusion protein with β-lactamase. The functionality of the purified fusion protein was tested *in vitro* in a prodrug converting cytotoxicity assay. In addition the immunoconjugate was tested *in vivo* in a tumor-targeting biodistribution study.

A non-limiting example of a therapeutic target against which a polypeptide construct of the invention may be used is *Helicobacter pylori*, which is a bacterium that lives in the mucus which coats the lining of the human stomach and duodenum. The normal human stomach has a very thin layer of mucus that coats the whole of its inside surface. This mucus has a protective role, acting as a barrier between the acid in the stomach and the sensitive stomach wall. *H. pylori* acts as an irritant to the lining of the stomach, and this causes inflammation of the stomach (gastritis). In one embodiment of the invention is a polypeptide construct comprising at least one single domain antibody directed against *H. pylori*, said construct and inhibits the enzymatic function of urease. Since single domain antibodies, in particular VHHs have the specific characteristic to occupy enzymatic sites, selected VHHs would inhibit the enzymatic activity and neutralize the virulence of a *H. pylori* infection. In another aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against *H. pylori* , said construct inhibiting the adhesion of the bacteria to the stomach wall so preventing irritation of the stomach wall and gastritis. One aspect of the invention is a polypeptide construct comprising one or more single domain antibodies directed against

Helicobacter pylori for use in the treatment, prevention and/or alleviation of disorders relating to irritation of the stomach wall and gastritis, wherein said polypeptide construct is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation, but preferably orally. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to irritation of the stomach wall and gastritis, comprising administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against *Helicobacter pylori*, orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation, but preferably orally.

Another non-limiting example of a therapeutic target against which the VHH of the invention may be used is Hepatitis E, which is a viral disorder transmitted via the fecal/oral route. Symptoms increase with age and include abdominal pain, anorexia, dark urine, fever, hepatomegaly, jaundice, malaise, nausea, and vomiting. The overall fatality rate is 1-3%, but 15-25% in pregnant women. Once encountered, most patients develop a neutralizing IgG response which gives life-long protection Neutralizing VHH molecules have the advantage over conventional IgG molecules because they may be administered orally. Since most infections with hepatitis E occur in North-Africa, Central-Africa, Asia and Central-America, oral administration is a significant advantage, since medical logistics are less developed in those countries. One aspect of the invention is one or more VHHs specific for HEV capsid protein (56kDa) for use in the treatment, prevention and/or alleviation of disorders relating hepatitis E, wherein said VHH is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to hepatitis E, comprising administering to a subject said VHH orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation."

Other non-limiting examples of therapeutic targets against which a polypeptide construct of the invention may be used are micro-organisms induce respiratory disorders such as the TB bacterium and influenza virus. TB or tuberculosis, is a disorder caused by bacteria called *Mycobacterium tuberculosis*. The bacteria can attack any part of the body, but they usually attack the lungs. Influenza is a viral disorder that causes 'flu'. Influenza viruses are also present in the lung. One aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against *Mycobacterium tuberculosis* epitope for use in

the treatment, prevention and/or alleviation of disorders relating TB, wherein said polypeptide construct is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to TB, comprising administering to a subject said polypeptide construct orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against an influenza virus epitope for use in the treatment, prevention and/or alleviation of disorders relating flu, wherein said polypeptide construct is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to flu, comprising administering to a subject said polypeptide construct orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation.

Another non-limiting example of a therapeutic target against which a polypeptide of the invention may be used is IgE in relation to allergies. During their lifetime, subjects may develop an allergic response to harmless parasites (e.g. Dermatophagoides pteronyssinus, house dust mite) or substances (clumps, plastics, metals). This results in the induction of IgE molecules that initiate a cascade of immunological responses. One aspect of the present invention is a polypeptide construct comprising at least one single domain antibody directed against IgE, said polypeptide preventing the interaction of IgE with their receptor(s) on mast cells and basophils. As such they prevent the initiation of the immunological cascade, an allergic reaction. Since IgE molecules are present in the bloodstream, it is within the scope of the invention to fuse the VHH one or more active transport carriers in order to reach their target. Another aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against an IgE epitope for use in the treatment, prevention and/or alleviation of disorders relating to allergies, wherein said polypeptide construct is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to allergies, comprising administering to a subject said polypeptide construct orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation.

According to one aspect of the invention, a polypeptide construct of the invention comprises at least one single domain antibody directed against IgE, said single domain antibody corresponding to a sequence represented by any of SEQ ID NOs: 1 to 11. Said sequences are anti-IgE *Camelidae* VHHs.

Another non-limiting example of a therapeutic target against which a polypeptide construct of the invention may be used is human macrophage elastase (MMP-12), which is a member of the family of matrix metalloproteases (MMPs). These enzymes play an important role in normal and inflammatory processes contributing to tissue remodeling and destruction. MMPs play besides proper extracellular matrix remodeling also an important role in diverse disease states such as cancer and inflammation. Macrophage elastase or MMP-12 has a large specificity pocket and broad substrate specificity. It plays a role in several disorders owing to excessive protein degradation of extracellular proteins (e.g. lung damage in smoke induced emphysema, Churg et al, A. 2003) or increased matrix degradation (e.g. higher MMP-12 enzymatic activity in obesity, Chavey et al, 2003). Other clinical indications include coeliac disorder and dermatitis herpetiformis (Salmela et al, 2001), glomerulo nephritis (Kaneko et al, 2003), esophageal squamous cell carcinoma (Ding et al, 2002) and skin cancer (Kerkela et al, 2000).

MMP-12 is secreted into the extracellular space by lung alveolar macrophages and dysregulation of MMP-12 is a possible reason for degradation of the alveolar membrane leading to lung emphysema. Target substrates of MMP-12 include extracellular matrix proteins such as elastin, fibronectin and laminin, but also α1-antitrypsin and tissue factor protease inhibitor. One aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against MMP-12 for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said polypeptide construct is administered orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to inflammatory processes, comprising administering to a subject said polypeptide construct orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation.

Another aspect of this invention consists of (1) VHH's that specifically bind to a metalloproteinase and are not degraded by a metalloproteinase, (2) VHH's which inhibit the proteolytic activity of one or more metalloproteinase and (3) inhibitory VHH's which are highly specific for one MMP (e.g. MMP-12 specific antagonist), unlike none-specific chemical inhibitors (e.g. batimastat, merimastat...)

According to one aspect of the invention, a polypeptide construct of the invention comprises at least one single domain antibody directed against human MMP-12, said single domain antibody corresponding to a sequence represented by any of SEQ ID NOs: 15 to 22. Said sequences are anti-MMP-12 *Camelidae* VHHs.

Another non-limiting example of a therapeutic target against which a polypeptide construct of the invention may be used is IFN-gamma, which is secreted by some T cells. In addition to its anti-viral activity, IFN gamma stimulates natural killer (NK) cells and T helper 1 (Th1) cells, and activates macrophages and stimulates the expression of MHC molecules on the surface of cells. Hence, IFN gamma generally serves to enhance many aspects of immune function, and is a candidate for treatment of disease states where the immune system is over-active (e.g. Crohn's disease), e.g., autoimmune disorders and organ plant rejection. One aspect of the invention is a polypeptide construct comprising at least one single domain antibody directed against IFN-gamma for use in the treatment, prevention and/or alleviation of disorders relating to the immune response, wherein said polypeptide construct is administered orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to the immune response, comprising administering to a subject said polypeptide construct orally, sublingually, topically, intravenously, subcutaneously, nasally, vaginally, rectally or by inhalation. In other embodiments of the present invention polypeptide constructs that neutralize IFN gamma are used to treat patients with psoriasis.

According to one aspect of the invention, a polypeptide construct of the invention comprises at least one single domain antibody directed against IFN-gamma, said single domain antibody corresponding to a sequence represented by any of SEQ ID NOs: 45 to 70. Said sequences are anti-IFN-gamma *Camelidae* VHHs.

The invention also relates to a method of identifying single domain antibodies (e.g. VHHs) harbouring specific sequences which facilitates the delivery or transport of the anti-target single domain antibodies across human or animal tissues (as described in US 6361938), including without limitation GIT epithelial layers, alveolar cells, endothelial of the blood-brain barrier, vascular smooth muscle cells, vascular endothelial cells, renal epithelial cells, M cells of the Peyers Patch, and hepatocytes. Furthermore, delivery systems could be used in conjunction with the VHH's of the invention, comprising nanoparticles, microparticles, liposomes, micelles, cyclodextrines. Only small (<600 daltons) and hydrophobic (Partridge et al, Adv. Drug Delivery Reviews, 15, 5-36 (1995)) molecules can easily pass the blood-brain barrier, severely limiting the development of novel brain drugs which can be used without the use of invasive neurosurgical procedures.

DELIVERING POLYPEPTIDE CONSTRUCTS TO THE INTERIOR OF CELLS

Another aspect of the present invention is a method and molecules for delivering therapeutic polypeptides and/or agents to the inside of cells. A further aspect of the invention is a method and molecules for delivering antigens to the inside of antigen presenting cells, and thereby eliciting a powerful immune response thereto. A still further aspect of the invention is to provide a method and molecules for delivery of therapeutic polypeptides and/or agents across natural barriers such as the blood-brain barrier, lung-blood barrier.

One aspect of the invention is a polypeptide construct comprising one or more single domain antibodies directed against a target and comprising one or more single domain antibodies directed against an internalising cellular receptor, wherein said polypeptide construct internalises upon binding to said receptor.

The targets inside cells may affect the functioning of said cell, or binding thereto may lead to a change in the phenotype of the cell itself by itself. This can be for example, cell death, effects on cell cycling or cell growth or interference with intracellular signaling pathways (see, for example, Poul MA et al, J Mol Biol, 2000, <u>301</u>, 1149-1161).

One embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies specific for an internalising cellular receptor, wherein said construct internalises upon binding to said receptor, wherein the polypeptide construct comprises a

therapeutic polypeptide or agent which is covalently or non-covalently linked thereto. Said therapeutic polypeptide or agent has one or more targets which acts intracellularly. See, for example, Figure 12. Said therapeutic polypeptides may harbour specific sequences which target the polypeptide to specific compartiments in the cell, comprising vesicles, organelles and other cytoplasmic structures, membrane-bound structures, the nucleus.

An internalising receptor according to the invention is a receptor displayed on the surface of a cell which upon binding to a ligand, mediates the internalisation of said ligand into the cytoplasm of the cell. Internalising receptors according to the invention include, but are not limited to, LDL receptors, EGFr, FGF2r, ErbB2r, transferrin receptor, PDGFr, VEGFr, PsmAr or antigen presenting cell internalising receptors.

One embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies specific for an internalising cellular receptor as disclosed herein, further comprising an antigen.

One embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies specific for an internalising cellular receptor as disclosed herein, wherein said receptor is an internalising receptor on an antigen presenting cell (APC). Preferably the receptor is highly specific for APCs and not present or is present in lower amounts on other cell types.

Another embodiment of the invention is a polypeptide construct comprising one or more anti-receptor single domain antibodies and an antigen. Thus by linking an antigen to a VHH directed towards an internalising receptor on an APC, antigen uptake by APC is not determined by the passive interaction between APC and antigen, but by the "active" binding between VHH and said receptor. This not only makes the process more efficient, but also more reproducible and not dependent on the antigen structure which causes great variability in the T-cell activation from antigen to antigen.

After internalization, the complex is digested by the APC and pieces of the antigen can be exposed on the surface in association with MHC/HLA and elicit a more powerful immune response.

Another embodiment of the present invention is a method for immunising a subject against an antigen comprising administering to a subject in need thereof a polypeptide construct comprising at least one single domain antibody directed against an antigen present on an APC, wherein said single domain antibody further comprises the antigen of interest.

One embodiment of the present invention is a polypeptide construct comprising one or more single domain antibodies specific for an internalising cellular receptor as disclosed herein, wherein said receptor is EGFR. In general internalization of receptors occurs upon binding of the agonistic ligand in a proces called sequestration. In order to ensure that extracellular signals are translated into intracellular signals of appropriate magnitude and specificity, the signalling cascades are tightly regulated via the process of sequestration, whereby receptors are physically removed from the cell surface by internalization to a cytosolic compartment (Carman, C.V. and Benovic, J.L. Current Opinion in Neurobiology 1998, 8: 335-344). This implies that only agonistic ligands or antibodies indeed are expected to internalize via such receptors. In terms of therapeutic use it is not a desired effect that the antibody first triggers proliferation of the tumorcells, before it can deliver a toxic payload to the interiour of the cell.

Some of internalising receptors are over-expressed on certain cells, such as the epidermal growth factor receptor (EGFR) or ErBb2 receptor on tumor cells. Epidermal growth factor (EGF) is an essential mediator of cell division in mammalian cells and is a recognized cellular oncogene and is therefore an appropriate target for anti-receptor therapy. After the binding of EGF to its receptor (EGFR), a signaling cascade is initiated resulting in cell development. The EGFR is involved in human tumorigenesis as it is overexpressed on cells of many epithelial malignancies such as head, neck, lung, colon. VHH that are internalised upon binding to one of these receptors can be used to deliver molecules inside the cell.

One embodiment of the present invention a polypeptide construct comprising one or more single domain antibodies directed against EGFR, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 23 to 44. Surprisingly, one of the single domain antibodies, did not activate the EGFR, despite the fact that it was internalized efficiently. Such types of antibodies are preferred for therapeutic applications, since these can deliver toxic payloads into cells without stimulating its proliferation.

Another embodiment of the present invention is a polypeptide construct construct comprising one or more single domain antibodies directed against for EGFR, wherein said anti-EGFR single domain antibody does not activate the EGFR. Said polypeptide construct may be used for the delivery of a therapeutic agents and/or polypeptides into a cell, as mentioned herein, without stimulating the EGFR.

Another embodiment of the present is a polypeptide construct construct comprising one or more single domain antibodies directed against for EGFR, wherein said anti-EGFR single domain antibody does not activate the EGFR and corresponds to a sequence represented by SEQ ID NO: 31.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, wherein said construct internalises upon binding to said receptor, and further comprising one or more single domain antibodies directed against an intracellular target, said single domain antibodies covalently or non-covalently linked. This multispecific polypeptide construct may be used in the treatment, prevention and/or alleviation of disorders, according to the target of the non-receptor specific single domain antibody. This target can be, for example, a kinase such as PDK1. PDK1 is over-expressed in breast tumor cells. It activates Akt by phosphorylating T308 in the activation loop. A number of downstream substrates of Akt play a direct role in promoting cell survival. These include GSK3, Bad, caspase-9 and Forkhead.

One embodiment of the present invention is a polypeptide construct comprising a single domain antibody directed against an internalising cellular receptor, wherein said construct internalises upon binding to said receptor, and further comprising one or more single domain antibodies directed against any of PDK1, GSK1, Bad, caspase-9 and Forkhead. Another aspect of the invention the use of said construct for treating cancer. Another aspect of the invention is said construct for the preparation of a medicament for treating cancer.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, wherein said construct internalises upon binding to said receptor, wherein the construct further comprises a drug or a toxic compound covalently or non-covalently linked thereto. One example of a

toxic compound is a compound that is only active intracellularly due to reducing environment (*e.g.* an enzyme recombinantly modified with additional cysteins resulting in inactive enzyme, but active in reducing environment). Another example of a toxic compound is a one that is specifically toxic only to a particular cell-type. An example of a toxic compound or a drug is a compound activated by a ligand present inside the cell and leading to the phenotype of interest. Other examples include prodrugs, small organic molecules. One aspect of the invention the use of said construct in the treatment of disorder requiring administration of the same. Another aspect of the invention is said construct for the preparation of a medicament for the treatment of disorder requiring administration of the same.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, wherein said construct internalises upon binding to said receptor, and wherein a filamentous phage expresses said construct on its surface. Said construct may be attached to the tip of the phage. In one aspect of the invention, construct-phage assembly can be used to package and deliver DNA to the cell for use as a gene therapy vector. According to the invention, the phage may carry DNA in additional to that encoding said construct, for use therapeutically. According to the invention, the phage may carry a gene encoding a therapeutic polypeptide controlled by a promoter for the expression of said gene inside the cell. An example of said promoter includes, but is not limited to, the CMV promoter (Kassner et al, Biochem Biophys Res Commun, 1999, 264: 921-928). Phage have distinct advantages over existing gene therapy vectors because they are simple, economical to produce at high titer, have no intrinsic tropism for mammalian cells, and are relatively simple to genetically modify and evolve (Larocca D *et al*, Curr. Pharm. Biotechnol, 2002: 3: 45-57).

Another embodiment of the present invention is a polypeptide construct as disclosed herein, wherein said single domain antibody is a peptide derived from a VHH specific for an internalising cellular receptor. Said VHH peptide may bind their antigen almost only through the peptide. Internalising VHHs may be prepared from a peptide library which is screened for internalising properties. It is an aspect of the invention that these VHH peptides can be added as a tag to therapeutic polypeptides or agents, for intracellular uptake. The VHH peptide, may, for example, be used to transport a therapeutic VHH into a cell. In one embodiment of

the invention, the VHH peptide is the CDR3. In another one embodiment of the invention, the VHH peptide is any other CDR.

Another embodiment of the present invention is a method of selecting for VHHs specific for an internalising cellular receptor, wherein said VHH internalise upon binding to said receptor, comprising panning receptor-displaying cells with a phage library (naïve or immune) of VHH, and selecting for internalising VHH by recovering the endocytosed phage from within the cell. The invention includes a selection method which uses cell lines that overexpress a receptor or cell lines transfected with a receptor gene to allow the easy selection of phage antibodies binding to the receptor. This avoids the need for protein expression and purification, speeding up significantly the generation of internalizing VHH.

Another embodiment of the present invention is a method for delivering a therapeutic polypeptide, agent or antigen for uptake by cellular internalisation by covalently or non-covalently attaching thereto a polypeptide construct comprising at least one single domain antibody specific for an internalising cellular receptor, wherein said construct internalises upon binding to said receptor.

The VHHs according to the invention may be used to treat, prevent and/or alleviate symptoms of disorders requiring the administration of the same.

Another embodiment of the present invention is a method for delivering a therapeutic polypeptide or agent that interacts with intracellular targets molecules comprising administering to a subject in need thereof one or more VHHs specific for an internalising cellular receptor, wherein said VHH internalise upon binding to said receptor, wherein said VHH is fused to said polypeptide or agent.

Another embodiment of the present invention is a method for delivering a therapeutic polypeptide, agent or antigen across a natural barrier by covalently or non-covalently attaching thereto a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, wherein said construct internalises upon binding to said receptor. According to the invention, a natural barrier includes, but is not

limited to, the blood-brain, lung-blood, gut-blood, vaginal-blood, rectal-blood and nasal-blood barriers.

For example, a peptide construct delivered via the upper respiratory tract and lung can be used for transport of therapeutic polypeptides or agents from the lung lumen to the blood. The construct binds specifically to a receptor present on the mucosal surface (bronchial epithelial cells) resulting in transport, via cellular internalisation, of the therapeutic polypeptides or agents specific for bloodstream targets from the lung lumen to the blood. In another example, a therapeutic polypeptide or agent is linked to a polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor present on the intestinal wall into the bloodstream. Said construct induces a transfer through the wall, via cellular internalization, of said therapeutic polypeptide or agent.

Another embodiment of the present invention is a VHH specific for an internalising cellular receptor, wherein said VHH internalises upon binding to said receptor, said VHH is covalently or non-covalently attached to a therapeutic polypeptide or agent, and said VHH crosses a natural barrier.

Another embodiment of the present invention is a method for delivering a therapeutic polypeptide, agent or antigen for uptake at a local by covalently or non-covalently attaching it to a VHH specific for an internalising cellular receptor, wherein said VHH internalises upon binding to said receptor. A local area, according to the invention, includes, but is not limited to, the brain, lung, gut, vaginal, rectal and nasal areas.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

As known by persons skilled in the art, once in possession of said polypeptide construct, formulation technology may be applied to release a maximum amount of VHHs in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating,

prevent and/or alleviate the symptoms of disorder whose targets that are located in the gut system.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder susceptible to modulation by a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies specific for antigen related to the disorder.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the prepararion of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the gut system without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by orally administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the vaginal and/or rectal tract.

In a non-limiting example, a formulation according to the invention comprises a polypeptide construct as disclosed herein comprising one or more VHHs directed against one or more targets in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time (such formulations are described in EP 707473, EP 684814, US 5629001).

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the vaginal and/or rectal tract without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the vaginal and/or rectal tract without being inactivated, by administering to the vaginal and/or rectal tract of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by administering to the vaginal and/or rectal tract of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target comprising at least one single domain antibody directed against a target, for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the nose, upper respiratory tract and/or lung.

In a non-limiting example, a formulation according to the invention, comprises a polypeptide construct as disclosed herein directed against one or more targets in the form of a nasal spray (e.g. an aerosol) or inhaler. Since the construct is small, it can reach its target much more effectively than therapeutic IgG molecules.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic delivered to the nose, upper respiratory tract and lung, by administering to a subject a polypeptide construct as disclosed herein wherein one or more single domain antibodies are specific for an antigen related to the disorder, by inhalation through the mouth or nose.

Another aspect of the invention is a dispersible VHH composition, in particular dry powder dispersible VHH compositions, such as those described in US 6514496. These dry powder compositions comprise a plurality of discrete dry particles with an average particle size in the range of 0.4-10 mm. Such powders are capable of being readily dispersed in an inhalation device. VHH's are particularly suited for such composition as lyophilized material can be readily dissolved (in the lung subsequent to being inhaled) due to its high solubilisation capacity (Muyldermans, S., Reviews in Molecular Biotechnology, 74, 277-303, (2001)). Alternatively, such lyophilized VHH formulations can be reconstituted with a diluent to generate a stable reconstituted formulation suitable for subcutaneous administration. For example, anti-IgE antibody formulations (Example 1; US 6267958, EP 841946) have been prepared which are usefull for treating allergic asthma.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the nose, upper respiratory tract and/or lung without being inactivated.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the nose, upper respiratory tract and lung, by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the nose, upper respiratory tract and/or lung without being inactivated, by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated by administering to the nose, upper respiratory tract and/or lung of a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

One embodiment of the present invention is a polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa. Because of their small size, a polypeptide construct as disclosed herein can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example, Crohn's disease.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa, by orally administering to a subject a polypeptide construct as disclosed herein.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound delivered to the intestinal mucosa, wherein said disorder increases the permeability of the intestinal mucosa.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the intestinal mucosa without being inactivated, by administering orally to a subject a polypeptide construct of the invention.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by administering orally to a subject a polypeptide construct of the invention.

This process can be even further enhanced by an additional aspect of the present invention - the use of active transport carriers. In this aspect of the invention, a polypeptide construct as described herein is fused to a carrier that enhances the transfer through the intestinal wall into the bloodstream. In a non-limiting example, this "carrier" is a VHH which is fused to said polypeptide. Such fusion constructs made using methods known in the art. The "carrier" VHH binds specifically to a receptor on the intestinal wall which induces an active transfer through the wall.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the tissues beneath the tongue effectively. A formulation of said polypeptide construct as disclosed herein, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capillary network under the tongue.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound that is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject a VHH specific for an antigen related to the disorder.

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able to pass through the tissues beneath the tongue.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the tissues beneath the tongue without being inactivated, by administering orally to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject without being inactivated, by administering orally to a subject a

polypeptide construct comprising one or more single domain antibodies directed against said target.

One embodiment of the present invention is a polypeptide construct comprising at least one single domain antibody for use in treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the skin effectively. A formulation of said polypeptide construct, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through.

An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by a therapeutic compound that is able pass through the skin effectively, by topically administering to a subject a polypeptide construct as disclosed herein comprising one or more single domain antibodies specific for an antigen related to the disorder.

Another aspect of the invention is the use of a polypeptide construct as disclosed herein as a topical ophthalmic composition for the treatment of ocular disorder, such as allergic disorders, which method comprises the topical administration of an ophthalmic composition comprising polypeptide construct as disclosed herein, said construct comprising one or more anti-IgE VHH (Example 1, Example 2).

Another embodiment of the present invention is a use of a polypeptide construct as disclosed herein for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of disorders susceptible to modulation by an anti-target therapeutic compound that is able pass through the skin effectively.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the skin without being inactivated, by administering topically to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

An aspect of the invention is a method for delivering an anti-target therapeutic compound to the bloodstream of a subject, by administering topically to a subject a polypeptide construct comprising one or more single domain antibodies directed against said target.

Another aspect of the present invention is a method to determine which single domain antibodies (e.g. VHHs) molecules cross a natural barrier into the bloodstream upon administration using, for example, oral, nasal, lung, skin. In a non-limiting example, the method comprises administering a naïve, synthetic or immune single domain antibody phage library to a small animal such as a mouse. At different time points after administration, blood is retrieved to rescue phages that have been actively transferred to the bloodstream. Additionally, after administration, organs can be isolated and bound phages can be stripped off. A non-limiting example of a receptor for active transport from the lung lumen to the bloodstream is the Fc receptor N (FcRn). The method of the invention thus identifies single domain antibodies which are not only actively transported to the blood, but are also able to target specific organs. The method may identify which VHH are transported across the gut and into the blood; across the tongue (or beneath) and into the blood; across the skin and into the blood etc.

One aspect of the invention are the single domain antibodies obtained by using said method. According to the invention, said single domain antibody may be used as a single domain antibody in a polypeptide construct of the invention. Said construct, further comprising another single domain antibody, a therapeutic agent, or polypeptide carrier directed against a target accessible via or in the blood may be administered by the route most efficient for said single domain antibody.

In general, “therapeutically effective amount”, “therapeutically effective dose” and “effective amount” means the amount needed to achieve the desired result or results (such as for instance modulating IFN-gamma binding; treating or preventing inflammation). One of ordinary skills in the art will recognize that the potency and, therefore, an “effective amount” can vary for the various compounds that modulate ligand-target binding, such as for instance IFN-gamma binding used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to a polypeptide construct of the present invention, or a nucleic acid capable of encoding said polypeptide construct.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The polypeptide constructs of the present invention are useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound or composition.

The polypeptide constructs as disclosed here in are useful for treating or preventing conditions in a subject and comprises administering a pharmaceutically effective amount of a compound combination with another, such as, for example, doxorubicin.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

A compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or a domestic animal in a variety of forms adapted to the chosen route of administration, *i.e.*, parenterally, intravenously, intramuscularly, subcutaneously, to the vaginal and/or rectal tract, nasally, by inhalation though the mouth or nose, to the tissues beneath the tongue, or topically.

A compound of the present invention can also be administered using gene therapy methods of delivery. See, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound may be administered in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be

incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, hydroxyalkyls or glycols or water-alcohol/glycol blends, in which the present compound can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances

and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compound to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compound can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the compound varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely

spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

BRIEF DESCRIPTION OF FIGURES

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Figure 4: Capacity of VHH clones to inhibit the proteolytic activity of human catalytic domain of MMP12

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Figure 12: Schematic illustrating a use of VHHs directed towards internalising receptors to deliver therapeutic protein, toxic compound, drug or polynucleotide.

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Table 5: Second round selection using neutravidine coated tubes as described in Example 1

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Examples:

IgE

Example 1: VHH directed against IgE

Two llama's were immunized with human IgE, Scripps laboratories, Cat nr. I0224. The following immunization schemes were used according to Table 1.

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987). cDNA was prepared on 200 µg total RNA with MMLV Reverse Transcriptase (Gibco BRL) using oligo d(T) oligonucleotides (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction, followed by an ethanol precipitation and subsequently used as template to amplify the VHH repertoire.

In a first PCR, the repertoire of both conventional (1.6 kb) and heavy-chain (1.3 kb) antibody gene segments were amplified using a leader specific primer (5'-GGCTGAGCTCGGTGGTCCTGGCT-3') and the oligo d(T) primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTTT-3'). The resulting DNA fragments were separated by agarose gel electrophoresis and the 1.3 kb fragment encoding heavy-chain antibody segments was purified from the agarose gel. A second PCR was performed using a mixture of FR1 reverse primers (WO03/054016 sequences ABL037 to ABL043) and the same oligo d(T) forward primer.

The PCR products were digested with *Sfi*I (introduced in the FR1 primer) and *Bst*EII (naturally occurring in framework 4). Following gel electrophoresis, the DNA fragments of approximately 400 basepairs were purified from gel and ligated into the corresponding restriction sites of

phagemid pAX004 to obtain a library of cloned VHHs after electroporation of *Escherichia coli* TG1. pAX004 allows the production of phage particles, expressing the individual VHHs as a fusion protein with a c-myc tag, a hexahistidine tag and the geneIII product. The percentage insert was determined in PCR using a combination of vector based primers. Results are summarized in Table 2.

Selections were done using chimaeric IgE instead of human IgE, used for immunization, in order to select for VHH molecules directed against the constant region of IgE. The region interacting with the Fcϵ-receptor is located in the constant part of IgE, more in particular in the region covered by Cϵ2-Cϵ3 as shown in Figure 1.

A first selection was performed using the pool of PBL day4, PBL day10 and lymph node day4 libraries for each of the two llama's. Chimaeric IgE was solid phase coated at 5 µg/ml and 0.5 µg/ ml and specific phages were eluted using 0.1 M glycine pH = 2.5. The results obtained are shown in Table 3.

A second selection was performed using the rescued phages from the first selection using 5 µg/ ml. Chimaeric IgE was solid phase coated at 1 µg/ml and specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy coat cells contain cells expressing the Fcϵreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 4.

Another second round selection was performed using neutravidine coated tubes and 2 nM biotinylated IgE. Specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy coat cells contain cells expressing the Fcϵreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 5.

Individual clones obtained from the first round of selection were screened in an ELISA using solid phase coated human IgE or chimaeric IgE. The number of clones that score positive for binding to both human IgE and chimeric IgE versus the number of clones tested in ELISA are summarized in Table 6.

Clones were picked which were positive for human and chimaeric IgE binding, amplified by PCR and digested with HinfI. HinfI profiles were determined on agarose gel and representative clones for different profiles were sequenced. The sequences obtained are shown in Table 14 SEQ ID NOs: 1 to 11.

Example 2: Topical applications of anti-IgE VHH's

To obtain anti-allergic pharmaceutical compositions for ophthalmic topical applications, a solution of anti-IgE VHH was prepared as follows:

- eye drops containing a therapeutic dose of anti-IgE VHH dissolved in 100 ml of sterilized water containing 0.9 g sodium chloride, 0.02 g sodium citrate, 0.02 g methyl parahydroxybenzoate, 0.1 g chlorobutanol and acetic acid suitabe to obtain a pH of 6.5.
- eye ointment containing a therapeutic dose of anti-IgE VHH was prepared according to the conventional method containing 1.0 g of liquid paraffin and a suitable amount of soft paraffin to obtain a total mixture of 100 g.

Example 3: Anti-IgE formulation

Anti-IgE VHH's that block binding of IgE to its high-affinity receptor are of potential therapeutic value in the treatment of allergy.

Highly purified VHH#2H11 was dialysed into formulation buffer, followed by addition of lyoprotectant at an isotonic concentration . Isotonic formulation was performed as follows: VHH#2H11 at 25mg/ml was formulated in 5mM histidine buffer at pH 6 with 500 moles of sugar per mole antibody. This formulation is reconstututed with BWFI (0.9% benzyl alcohol) at a volume which results in a 100 mg/ml of antibody in 20 mM histidine at pH 6 with an isotonic sugar concentration of 340 nM. The binding activity of the anti-IgE VHH in the isotonic formulations was measured in an IgE receptor inhibition assay. It was found that binding activity was essentially unchanged following storage at 4°C for up to 3 months.

<u>TNF-alpha</u>

Example 4: Selection of anti-TNF-alpha

Two llamas were immunized with 100 µg human TNF-alpha per injection according to the schedule described in Example 1. The libraries (short and long immunization procedure) were

constructed and selected with *in vitro* biotinylated TNF-alpha. The biotinylation was carried out as described by Magni et al (Anal Biochem 2001, 298, 181-188).

The incorporation of biotin in TNF was evaluated by SDS-PAGE analysis and detection with Extravidin-alkaline phosphatase conjugate (Sigma).

The functionality of the modified protein was evaluated for its ability to bind to the solid phase coated recombinant a p75 receptor. {biotinylation} In the first round of selection 400 ng and 50 ng of biotinylated TNF-alpha was captured on neutravidin (Pierce; 10 µg/ml in PBS) coated on the wells of a microtiter plate (NUNC maxisorb). Phage (1.2×10^{10} TU-s) were added to the wells and incubated for two hours at room temperature. After washing (20 times with PBS-tween and two times with PBS) bound phage was eluted by adding an excess of receptor (extracellular domain of CD120b or p75; 10 µM) or with cells expressing the intact TNF receptor. Between 30,000 and 100,000 phage clones were eluted with TNF from the library derived from the llama immunized using the rapid scheme, while about 10% of these numbers were obtained when eluted with BSA (3 µM; negative control).

From the other library (long immunization scheme) 10-fold high numbers were eluted with receptor and BSA, yielding the same enrichment factor (10) as observed before. New phage was prepared from the elution of 50 ng TNF (rapid immunization scheme) and 400 ng TNF (slow scheme) and used for another round of selection on 400, 50 and 10 ng of captured TNF (input: 1.2×10^{10} phage per well). Approx. 2.5×10^{7} phage were eluted with receptor (10 µM) from the well containing 400 ng and 50 ng of captured TNF and about 2×10^{6} from the well with 10 ng of TNF, while the negative control (elution with 10 µM of BSA) gave only 5 to 10% of those numbers. The observed numbers of eluted phage suggest that the elution with receptor is specific and that those VHH fragments should be eluted that bind to the receptor binding site of TNF.

Individual clones were picked and grown in microtiter plate for the production of VHH in culture supernatants. ELISA screening with TNF captured on Extravidin coated plates revealed about 50% positive clones. HinFI-fingerprint analysis showed that 14 different clones were selected, which were grown and induced on 50 ml scale.

Periplasmic fractions were prepared, the VHH fragments purified with IMAC and used in an assay to analyze their antagonistic characteristics, i.e. preventing the interaction of TNF with its receptor. For this purpose the VHH (1 μM and 0.3 μM) was incubated with TNF-alpha (3 and 0.7 nM) for 1.5 hours at room temperature (in 0.2% casein / PBS). 100 μl of this mixture was transferred to a well of a microtiter plate, in which the extracellular domain of the receptor was immobilized. After an incubation of one hour the plate was washed and bound TNF was detected with alkaline phosphatase conjugated streptavidin. Two VHH fragments gave antagonistic profiles similar as obtained with 3 and 0.3 μM intact mAB Remicade (Infliximab; Centercor) in spite of the fact that the VHH is truly monomeric, whereas the dimeric appearance of the mAB probably favors the binding of the trimeric TNF-molecule. Similar experiments showing the efficacy of the VHH were performed using the murine sarcoma cell line WEHI and a human cell line expressing the TNF receptor. The sequences obtained are shown in Table 14 SEQ ID NOs: 12 to 13.

Example 5: Stability testing of antibody fragments specific for human TNFα

Orally administered proteins are subject to denaturation at the acidic pH of the stomach and as well to degradation by pepsin. We have selected conditions to study the resistance of the VHH TNF3E to pepsin which are supposed to mimick the gastric environment. TNF3E a VHH specific to human TNFα was produced as recombinant protein in E.coli and purified to homogeneity by IMAC and gelfiltration chromatography. The protein concentration after purification was determined spectrophotometrically by using the calculated molar exctinction coefficient at 280nm. Diluted solutions at 100 microgram/ml were prepared in McIlvaine buffer (J. Biol. Chem. 49, 1921, 183) at pH 2, pH3 and 4 respectively. These solutions were subsequently incubated for 15 minutes at 37°C, prior the addition of porcine gastric mucosa pepsin at a 1/30 w/w ratio. Sixty minutes after adding the protease a sample was collected and immediately diluted 100-fold in PBS pH7.4 containing 0.1% casein to inactivate the pepsin. Seven additional 3-fold dilutions were prepared from this sample for assessing the presence of functional antibody fragment by ELISA. Identical dilutions prepared from an aliquot collected prior the addition of the protease served as a reference. In the ELISA assay biotinylated TNFα was captured in wells of a microtiter plate coated with neutravidin. For both the pepsin-treated and reference samples similar serial dilutions of the samples were prepared and 100 microliter of those dilutions were added to the wells. After incubation for 1 hour the plates were washed. For the detection of VHH binding to of the captured TNFα a

polyclonal rabbit anti-VHH antiserum (R42) and an anti-rabbit IgG alkaline phosphatase conjugate was used. After washing, the plates were developed with para nitrophenyl phosphate. The data plotted in Figure 2 shows similar curves for all of the samples exposed to digestive conditions as well as for the reference samples. This indicates that the VHH 3E essentially retains its functional activity under all of the chosen conditions.

Example 6: Oral administration of an anti-human TNFα specific VHH in mice

An antibody solution containing the anti-human TNFα specific VHH#TNF3E (100microgram per milliliter in 100-fold diluted PBS) was prepared. Three mice which were first deprived from drinking water for 12 hours and subsequently allowed to freely access the antibody solution during the next two hours. Afterwards the mice were sacrificed and their stomachs were dissected. Immediately the content of the stomachs was collected by flushing the stomach with 500microliter PBS containing 1% BSA. This flushed material was subsequently used to prepare serial three-fold dilutions, starting at a 1/5 dilution from the undiluted material. One hundred microliter of these samples was transferred to individual wells of a microtiter plater coated with human TNFα. After incubation for 1 hour and following extensive washing the presence of immuno-reactive material was assessed with a polyclonal rabbit anti-VHH antiserum (R42) followed by incubation with an anti-rabbit alkaline-phosphatase conjugate.The ELISA was developed with paranitrophenyl phosphate. The ELISA signals obtained after 10 minutes clearly demonstrated the presence of functional VHH TNF3E in the gastric flushings of these mice. By comparing to the standard curve we determined the concentration of the functional antibody fragment in the gastric flushing fluid to be 1.5, 12.6 and 8.6 microgram/ml for the three mice tested.

Example 7: Efficacy in an animal model for IBD

1) Animal model of chronic collitis

The efficacy of bivalent VHH constructs applied via various routes of administration was assessed in a DSS (dextran sodium sulfate) induced model of chronic colitis in BALB/c mice. This model was originally described by Okayasu et al. [Okayasu et al. Gastroenterology 1990; **98**: 694-702] and modified by Kojouharoff et. al. [G. Kojouharoff et al. Clin. Exp. Immunol. 1997; **107**: 353-8]. The animals were obtained from Charles River Laboratories, Germany, at an age of 11 weeks and kept in the animal facility until they reached a body weight between 21 and 22 g. Chronic colitis was induced in the animals by four DSS

treatment cycles. Each cycle consisted of a DSS treatment interval (7 days) where DSS was provided with the drinking water at a concentration of 5 % (w/v) and a recovery interval (12 days) with no DSS present in the drinking water. The last recovery period was prolonged from 12 to 21 days to provide for an inflammation status rather representing a chronic than an acute inflammation at the time of the treatment. Subsequent to the last recovery interval the mice were randomly assigned to groups of 8 mice and treatment with the VHH-constructs was started. The treatment interval was 2 weeks. One week after the end of the treatment interval the animals were sacrificed, the intestine was dissected and histologically examined. The experimental setting is shown schematically in Figure 3.

2) <u>VHH treatment schedule</u>

During the VHH treatment period the mice (8 animals per group) were treated daily for 14 consecutive days with bivalent VHH#3F (VHH#3F-VHH#3F; SEQ ID No. 14) by intra-gastric or intra-venous application of 100 µg bivalent VHH 3F. An additional group of animals was treated rectally with the bivalent VHH#3F every other day for a period of 14 days. In all treatment groups a dose of 100 µg of the bivalent VHH#3F was applied at a concentration of 1 mg/ml in a buffered solution. The negative control groups received 100 µl of PBS under otherwise identical conditions. The treatment schedule is shown in Table 7.

3) <u>Results</u>

After the mice were sacrificed the body weight was determined and the colon was dissected. The length of the dissected colon was determined and the histology of the colon was assessed by Haematoxilin-Eosin (HE) stain (standard conditions). As compared to the negative controls (PBS treatment) the groups treated with bivalent nanobody 3F showed a prorogued colon length as well as an improved histological score [G. Kojouharoff et al. Clin. Exp. Immunol. 1997; **107**: 353-8] thereby demonstrating efficacy of the treatment.

<u>**MMP12**</u>

Example 8: Immunization

One llama's (llama 5) was immunized intramuscularly with recombinant human catalytic domain of MMP12 using an appropriate animal-friendly adjuvant Stimune (Cedi Diagnostics BV, The Netherlands). The recombinant catalytic domain was acquired from Prof. H.

Tschesche Universität Bielefeld and was supplied as a 56µg/ml solution in 5mM Tris/HCl pH=7.5, 100mM NaCl, 5mM $CaCl_2$ (Lang, R. et al. (2001). The llama received 6 injections at weekly intervals, the first two injections containing each 10 µg of MMP-12, the last four injections containing each 5 µg of MMP-12. Four days after the last immunization a lymph node biopsy (LN) and a blood sample (PBL1) of 150ml was collected from the animal and serum was prepared. Ten days after the last immunization a second blood sample (PBL2) of 150ml was taken and serum was prepared. Peripheral blood lymphocytes (PBLs), as the genetic source of the llama heavy chain immunoglobulins (HcAbs), were isolated from the blood sample using a Ficoll-Paque gradient (Amersham Biosciences) yielding $5x10^8$ PBLs. The maximal diversity of antibodies is expected to be equal to the number of sampled B-lymphocytes, which is about 10 % of the number of PBLs ($5x10^7$). The fraction of heavy-chain antibodies in llama is up to 20 % of the number of B-lymphocytes. Therefore, the maximal diversity of HcAbs in the 150 ml blood sample is calculated as 10^7 different molecules. Total RNA was isolated from PBLs and lymph nodes according to the method of Chomczynski and Sacchi (1987).

Example 9: Repertoire cloning

cDNA was prepared on 200 µg total RNA with MMLV Reverse Transcriptase (Gibco BRL) using oligo d(T) oligonucleotides (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction, followed by an ethanol precipitation and subsequently used as template to amplify the VHH repertoire.

In a first PCR, the repertoire of both conventional (1.6 kb) and heavy-chain (1.3 kb) antibody gene segments were amplified using a leader specific primer (5'-GGCTGAGCTCGGTGGTCCTGGCT-3') and the oligo d(T) primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTT-3'). The resulting DNA fragments were separated by agarose gel electrophoresis and the 1.3 kb fragment encoding heavy-chain antibody segments was purified from the agarose gel. A second PCR was performed using a mixture of FR1 reverse primers (WO03/054016 sequences ABL037 to ABL043) and the same oligo d(T) forward primer.

The PCR products were digested with *Sfi*I (introduced in the FR1 primer) and *BstE*II (naturally occurring in framework 4). Following gel electrophoresis, the DNA fragments of approximately 400 basepairs were purified from gel and ligated into the corresponding restriction sites of phagemid pAX004 to obtain a library of cloned VHHs after electroporation of *Escherichia coli*

TG1. pAX004 allows the production of phage particles, expressing the individual VHHs as a fusion protein with a c-myc tag, a hexahistidine tag and the geneIII product. The diversity obtained after electroporation of TG1 cells is presented in Table 8. The percentage insert was determined in PCR using a combination of vector based primers.

Example 10: Rescue of the library and phage preparation

The library was grown at 37°C in 10 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD_{600nm} reached 0.5. M13KO7 phages (10^{12}) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 5 minutes at 4,500 rpm at room temperature. The bacterial pellet was resuspended in 50 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 37°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 4,500 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) for 30 minutes on ice and centrifuged for 20 minutes at 4,500 rpm. The pellet was resuspended in 1 ml PBS. Phages were again PEG precipitated for 10 minutes on ice and centrifuged for 10 minutes at 14,000 rpm and 4°C. The pellet was dissolved in 1 ml 0.5% skimmed milk or PBS-BSA [1mg/ml] (Sigma, Cat Nr A3059).

Example 11: Selection of human MMP-12 specific VHH

Phages were rescued and prepared as described above in Example 10.

Two approaches were followed to obtain MMP-12 specific binders:

a. <u>Inactive MMP-12 coated on PVDF Membrane</u>

100ng human MMP-12 catalytic domain (diluted in 33µl PBS) was spotted on small pieces ($1cm^2$) of PVDF (Immobilon-P, Millipore, Cat Nr IPVH 15150) following the manufacturers guidelines, resulting in an inactive MMP due to the MeOH fixation. As controls an equal amount of lysozyme (Sigma, Cat Nr L-6876) and 33µl PBS were also spotted and immobilized. The membrane pieces were blocked overnight in 5% skimmed milk at 4°C and were washed 3 times with PBS before the phage preparation was applied ($4x10^9$ phages in 1ml [5% skimmed milk]). Phages and membrane pieces (in 1,5ml tubes) were incubated for 3 hrs at room temperature with rotation. Then the membranes were transferred to 15ml tubes and were washed 6 times with 10ml [PBS+0.05%Tween-20].

Phages were eluted by exposing the membranes to 500µl TEA [70µl in 5ml H_2O] for 10min while rotating. The solution containing the eluted phages was removed and the pH was neutralized with 1M Tris pH=7.5.

Log phase growing TG1 cells were infected with the eluted phages and serial dilutions were plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection obtained from the MMP-12 coated membrane as compared with the negative control where lysozyme was immobilized. Bacteria from MMP selections showing enrichment were scraped and used for a second round of selection.

The bacteria were superinfected with helperphage to produce recombinant phages to do a second selection against MMP-12 (as described in Example 9). MMP-12 was immobilized as above and the membrane was blocked overnight at 4°C in 5% skim milk. Phages ($2.5x10^9$ in 1ml) were prepared and exposed to the membranes and further selected for MMP binding as during the first round of selection. Log phase growing TG1 cells were infected with the eluted and pH neutralized phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies from the MMP-12 coated membrane as compared with the negative control (immobilized lysozyme).

b. <u>Active MMP-12 coated on Nitrocellulose Membrane</u>

250ng human MMP-12 catalytic domain (Biomol Research laboratories Inc, SE 138-9090) was spotted directly on a piece of Hybond-C extra (Amersham Biosciences, Cat Nr RPN 303E) following the suppliers guidelines. As control an equal volume of PBS was spotted. A 5mm diameter disk, containing the spotted area was cut out from each membrane and was transferred to a 1.5ml tube and blocked overnight at 4°C in 1ml BSA-PBS [1mg/ml]. The disks were washed three times in 15ml PBS and subsequently transferred and exposed to the 200ul phage preparation in a microtiterplate well. The phages were prepared as in Example 9 but were preincubated in BSA-PBS for 15min at room temperature. The disks were washed 5 times with PBS/0.05%Tween-20 and were blocked with PBS-BSA for 2 hrs at room temperature. Phages were eluted by exposing the membranes to 100µl TEA [70µl in 5ml H_2O] for 10min while rotating. The solution containing the eluted phages was removed and the pH was neutralized with 1M Tris pH=7.5.

Log phase growing TG1 cells were infected with the eluted phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection on the MMP-12 membrane disk as compared with the negative control (PBS). Bacteria from selections with MMP-12 were scraped and used for a second round of selection.

The bacteria were superinfected with helperphage to produce recombinant phages to do a second selection against MMP-12 (as described in Example 9). MMP-12 was immobilized as above and the membrane was blocked overnight at 4°C in PBS-BSA [1mg/ml]. Phages (2.5×10^9 in 1ml) were prepared and exposed to the membranes and further selected for MMP binding as during the first round of selection. Log phase growing TG1 cells were infected with the eluted and neutralized phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies from the MMP-12 coated membrane as compared with the negative control.

Example 12: Specificity of selected VHH's

Individual clones were picked, grown in 150 µl 2xTY containing 0.1% glucose and 100 µg/ml ampicillin in a microtiter plate at 37°C until OD_{600nm}= 0.6. Then 1 mM IPTG and 5 mM $MgSO_4$ was added and the culture was incubated 4 hours at 37°C. ELISA was performed on the periplasmic extracts (PE, preparation see Example 13) of the cells to examine specificity of the selected clones.

To examine the clones selected using solid phase coated human MMP-12, plates were coated with human MMP-12 catalytic domain at a concentration of 1µg/ml overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Wells were blocked with 1% skimmed milk for 2 hrs at room temperature. Periplasmic extracts (100µl) were applied to the wells and incubated for 1 hour at room temperature. Plates were washed 5 times with PBS/0.05%Tween-20. Detection was performed using anti-c-myc antibody, followed by anti-mouse-HRP and ABTS/H_2O_2 as substrate. Plates were read at 405nm after 30 minutes incubation at room temperature.

To examine the clones selected using membrane immobilized human MMP-12, 50ng human MMP-12 catalytic domain samples were spotted on PVDF membrane as described in the manufacturers guidelines. 50ng lysozyme was spotted as a negative control. The membranes were blocked with skimmed milk overnight at 4°C, washed 5 times with PBS and transferred to 1,5ml tubes. Periplasmic extracts (100µl) were tenfold diluted in 1% skimmed milk and 1ml

was applied per membrane ($2cm^2$) and rotated for 1 hour at room temperature. Membranes were washed 5 times with PBS/0.05%Tween-20. Detection was performed using anti-c-myc antibody, followed by anti-mouse-HRP and DAP as substrate. Membranes were incubated with substrate at room temperature until clear spots were visible. Seven clones which were found to be MMP-12 specific binders are shown in Table 14 SEQ ID NOs 15 to 21.
In order to check for non specific binding to other MMPs a similar approach was followed in which 50ng of active catalytic domain of MMP 1, 2, 3, 7, 9 and 13 (all from Biomol Research laboratories Inc) was immobilized on Hybond C-extra. The membranes were blocked with skimmed milk overnight at 4°C, washed 5 times with PBS and transferred to 1,5ml tubes. Periplasmic extracts (100μl) were tenfold diluted in 1% skimmed milk and 1ml was applied per membrane ($2cm^2$) and rotated for 1 hour at room temperature. Membranes were washed 5 times with PBS/0.05%Tween-20. Detection was performed using anti-c-myc antibody, followed by anti-mouse-HRP and DAP as substrate. Membranes were incubated with substrate at room temperature until clear spots were visible. No significant detection of the seven selected VHH clones was observed on any of the MMPs other than MMP-12.

Results on binders selected against PVDF membrane immobilized human MMP-12 catalytic domain are presented in Table 14 SEQ ID NOs 15 to 21.
Results on MMP-12 inhibitors selected via Hybond membrane immobilization are presented in Table 14 SEQ ID NO 22.

Example 13: Diversity of selected VHH's

PCR was performed using M13 reverse and genIII forward primers. The clones were analyzed using Hinf1 fingerprinting and representative clones were sequenced. Sequence analysis was performed resulting in the sequences which are presented in Table 14 SEQ ID NOs 15 to 21 for Immobilon-P selections and in Table 14 SEQ ID NO 22 for Hybond-C.

Example 14: Expression and purification of VHH

Clones were grown in 50 ml 2xTY containing 0.1% glucose and 100 μg/ml ampicillin in a shaking flask at 37°C until OD_{600nm}= 2. 1 mM IPTG and 5 mM $MgSO_4$ was added and the culture was incubated for 3 more hours at 37°C. Cultures were centrifuged for 10 minutes at 4,500 rpm at 4°C. The pellet was frozen overnight at -20°C. Next, the pellet was thawed at room temperature for 40 minutes, re-suspended in 1 ml PBS/1mM EDTA/1M NaCl and shaken

on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 10 minutes at 4°C at 4,500 rpm. The supernatant containing the VHH was loaded on Ni-NTA (Qiagen) and purified to homogeneity on an Äkta FPLC chromatography system (Amersham Biosciences). The VHH were eluted from the Ni-NTA using 25mM citric acid pH=4,0 and directly applied on a cation exchange column equilibrated in 25mM citric acid pH=4,0 (Source 30S in a HR5/5 column, Amersham Biosciences). The VHH were eluted with 1M NaCl in PBS and further purified on a size exclusion column (Superdex 75 HR10/30, Amersham Biosciences) equilibrated in MMP-12 assay buffer [50mM HEPES, 100mM NaCl, 0,05%Brij-35]. The yield of VHH was calculated according to the extinction coefficient and peak surface area.

Example 15: Functional characterization of selected VHH's: inhibition of MMP-12 proteolytic activity by a VHH in a colorimetric assay.

VHHs were expressed and purified as described in Example 13. Purified VHH was analyzed for the ability to inhibit human MMP-12 catalytic domain using the MMP-12 Colorimetric Assay Kit for Drug Discovery (AK-402) from BIOMOL Research Laboratories. The experimental method conditions described in the Kit were followed.

The inhibitor supplied with the Kit (PI115-9090) was used as positive control at the recommended concentration. VHH were applied at a concentration of 7μM. The assay was performed in the microtiterplate supplied with the BIOMOL Kit and MMP-12 proteolytic activity was followed in a plate reader (405nm) at 37°C.

The results of one inhibitory VHH and an inactive VHH are presented in Figure 4 together with a positive control.

Only one VHH molecule (clone P5-29) from selections using active MMP-12 coated on nitrocellulose (Example 12) showed inhibition of human MMP-12 catalytic domain. All other MMP-12 binders (only clone P5-5 is shown), although they bind MMP-12, did not inhibit MMP-12.

Example 16: Formulation of anti-MMP12 VHH for pulmonary delivery

A 100% formulation of antibody was prepared by dissolving 5 mg of VHH in 1.0 ml of deionized water. The pH of the solution was 6.5. A 90% formulation of antibody was prepared by dissolving 4.5 mg of VHH in 1.0 ml of 2mM citrate buffer. A 70% formulation of antibody was prepared by dissolving 3.5 mg of VHH in 1 mg/ml of excipient in 1 ml of citrate buffer at pH 6.5. The various classes of excipients used were as follows: Sugar excipients: sucrose,

lactose, mannitol, raffinose and trehalose. Polymeric excipients: ficoll and PVP. Protein excipients: HSA.

Dry powders of the above formulations were produced by spray drying using a Buchi Spray Dryer.

The particle size distribution was measure by centrifugal sedimentation.

Interferon-gamma

Example 17: Immunization

Four llama's (llama 5, 6, 22 and 23) were immunized intramuscularly with human IFN-γ (PeproTech Inc, USA, Cat Nr: 300-02) using an appropriate animal-friendly adjuvant Stimune (Cedi Diagnostics BV, The Netherlands). Two llama's (llama 29 and 31) were immunized intramuscularly with mouse IFN-γ (Protein Expression & Purification core facility, VIB-RUG, Belgium) using an appropriate animal-friendly adjuvant Stimune (Cedi Diagnostics BV, The Netherlands). The llama's received 6 injections at weekly intervals, the first two injections containing each 100 μg of IFN-γ, the last four injections containing each 50 μg of IFN-γ. Four days after the last immunization a blood sample (PBL1) of 150ml and a lymph node biopsy (LN) was collected from each animal and sera were prepared. Ten days after the last immunization a second blood sample (PBL2) of 150ml was taken from each animal and sera were prepared. Peripheral blood lymphocytes (PBLs), as the genetic source of the llama heavy chain immunoglobulins (HcAbs), were isolated from the blood sample using a Ficoll-Paque gradient (Amersham Biosciences) yielding $5x10^8$ PBLs. The maximal diversity of antibodies is expected to be equal to the number of sampled B-lymphocytes, which is about 10 % of the number of PBLs ($5x10^7$). The fraction of heavy-chain antibodies in llama is up to 20 % of the number of B-lymphocytes. Therefore, the maximal diversity of HcAbs in the 150 ml blood sample is calculated as 10^7 different molecules. Total RNA was isolated from PBLs and lymph nodes according to the method of Chomczynski and Sacchi (1987).

Example 18: Repertoire cloning

cDNA was prepared on 200 μg total RNA with MMLV Reverse Transcriptase (Gibco BRL) using oligo d(T) oligonucleotides (de Haard *et al.*, 1999). The cDNA was purified with a phenol/chloroform extraction, followed by an ethanol precipitation and subsequently used as template to amplify the VHH repertoire.

In a first PCR, the repertoire of both conventional (1.6 kb) and heavy-chain (1.3 kb) antibody gene segments were amplified using a leader specific primer (5'-GGCTGAGCTCGGTGGTCCTGGCT-3') and the oligo d(T) primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTT-3'). The resulting DNA fragments were separated by agarose gel electrophoresis and the 1.3 kb fragment encoding heavy-chain antibody segments was purified from the agarose gel. A second PCR was performed using a mixture of FR1 reverse primers (WO03/054016 sequences ABL037 to ABL043) and the same oligo d(T) forward primer.

The PCR products were digested with *Sfi*I (introduced in the FR1 primer) and *BstE*II (naturally occurring in framework 4). Following gel electrophoresis, the DNA fragments of approximately 400 basepairs were purified from gel and ligated into the corresponding restriction sites of phagemid pAX004 to obtain a library of cloned VHHs after electroporation of *Escherichia coli* TG1. pAX004 allows the production of phage particles, expressing the individual VHHs as a fusion protein with a c-myc tag, a hexahistidine tag and the geneIII product. The diversity obtained after electroporation of TG1 cells is presented in Table 1. The percentage insert was determined in PCR using a combination of vector based primers.

Example 19: Rescue of the library and phage preparation

The library was grown at 37°C in 10 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD_{600nm} reached 0.5. M13KO7 phages (10^{12}) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 5 minutes at 4,500 rpm at room temperature. The bacterial pellet was resuspended in 50 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 37°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 4,500 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) for 30 minutes on ice and centrifuged for 20 minutes at 4,500 rpm. The pellet was resuspended in 1 ml PBS. Phages were again PEG precipitated for 10 minutes on ice and centrifuged for 10 minutes at 14,000 rpm and 4°C. The pellet was dissolved in 1 ml PBS-0.1% casein.

Example 20: Selection of human IFN-γ specific VHH

Phages were rescued and prepared as described above in example 17

Two approaches were followed to obtain IFN-γ specific binders:

a. Solid phase coated IFN-γ

Microtiter wells were coated with human IFN-γ at different concentrations of 10-0.4 μg/well overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Wells were blocked with PBS+1% caseine for 2 hrs at room temperature. Phages were incubated for 2 hrs at room temperature. Wells were washed 20 times with PBS+0.05%Tween-20. The two final washes were performed using PBS. Specific phages were eluted using 1 to 2 μg of IFN-γ R1 (R&D Systems, Cat Nr: 673-IR/CF) for 1 hr. As negative control elutions were performed using 10 μg Ovalbumine (Sigma, A2512) as irrelevant protein. Log phase growing TG1 cells were infected with the eluted phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection using the receptor for elution as compared with negative control using ovalbumine for elution. Bacteria from selections showing enrichment were scraped and used for a second round of selection.

The bacteria were superinfected with helperphage to produce recombinant phages as described in example 3. Microtiter wells were coated with IFN-γ at different concentrations of 2-0.1 μg/well overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Wells were blocked with PBS+1% caseine for 2 hrs at room temperature. Phages were incubated for 2 hrs at room temperature. Wells were washed 20 times with PBS+0.05%Tween-20. The two final washes were performed using PBS. Specific phages were eluted using 1 to 2 μg of IFN-γ R1 or 10 μg Ovalbumine as irrelevant protein for 1 hr, subsequently overnight at 4°C and subsequently, phages were eluted using 0.1 M glycine pH 2.5 for 15 minutes at room temperature and neutralized with 1M Tris-HCl pH=7.5. Log phase growing TG1 cells were infected with the eluted and neutralized phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection using the receptor for elution as compared with negative control using ovalbumine for elution.

b. Biotinylated IFN-γ

Microtiter wells were coated with neutravidine at a concentration of 2 µg/ml overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Wells were blocked with PBS+1% caseine for 2 hrs at room temperature. Biotinylated human IFN-γ at a concentration of 100-10 ng/well was captured overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Phages were incubated for 2 hrs at room temperature. Wells were washed with PBS+0.05%Tween-20. The two final washes were performed using PBS. Specific phages were eluted using 1 to 2 µg of IFN-γ R1 (R&D Systems, Cat Nr: 673-IR/CF) for 1 hr. As negative control elutions were performed using 10 µg Ovalbumine (Sigma, A2512) as irrelevant protein. Log phase growing TG1 cells were infected with the eluted phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection using the receptor for elution as compared with negative control using ovalbumine for elution. Bacteria from selections showing enrichment were scraped and used for a second round of selection.
Bacteria were superinfected with helperphage to produce recombinant phages. Microtiter wells were coated with neutravidine at a concentration of 2 µg/ml overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Wells were blocked with PBS+1% caseine for 2 hrs at room temperature. Biotinylated human IFN-γ at a concentration of 20-2.5 ng/100 µl was captured overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20. Phages were incubated for 2 hrs at room temperature. Wells were washed 20 times with PBS+0.05%Tween-20. The two final washes were performed using PBS. Specific phages were eluted using 1 to 2 µg of IFN-γ R1 or 10 µg Ovalbumine as irrelevant protein for 1 hr, subsequently overnight at 4°C and subsequently, phages were eluted using 0.1 M glycine pH 2.5 for 15 minutes at room temperature and neutralized with 1M Tris-HCl pH=7.5. Log phase growing TG1 cells were infected with the eluted and neutralized phages and plated on selective medium. Enrichment was determined by the number of transfected TG1 colonies after selection using the receptor for elution as compared with negative control using ovalbumine for elution.

Example 21: Diversity of selected VHH's

PCR was performed using M13 reverse and genIII forward primers. The clones were analyzed using Hinf1 fingerprinting and representative clones were sequenced. Sequence

analysis was performed resulting in the sequences presented in Table 4 for human IFN-γ (SEQ ID No. 45-70).

Example 22: Expression and purification of VHH

Small scale expressions were started after transformation of DNA into WK6 *Escherichia coli* cells.

Clones were grown in 50 ml 2xTY containing 0.1% glucose and 100 µg/ml ampicillin in a shaking flask at 37°C until OD_{600nm}= 2. 1 mM IPTG and 5 mM $MgSO_4$ was added and the culture was incubated for 3 more hours at 37°C. Cultures were centrifuged for 10 minutes at 4,500 rpm at 4°C. The pellet was frozen overnight at -20°C. Next, the pellet was thawed at room temperature for 40 minutes, re-suspended in 1 ml PBS/1mM EDTA/1M NaCl and shaken on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 10 minutes at 4°C at 4,500 rpm. The supernatant containing the VHH was loaded on TALON (Clontech) and purified to homogeneity. The yield of VHH was calculated according to the extinction coefficient.

Example 23: Topical applications of anti-IFN gamma VHH's

1: To obtain anti-allergic pharmaceutical compositions for ophthalmic topical applications, a solution of at least one anti-IFN gamma VHH was prepared as follows:

- eye drops containing a therapeutic dose of anti-IFN gamma VHH dissolved in 100 ml of sterilized water containing 0.9 g sodium chloride, 0.02 g sodium citrate, 0.02 g methyl parahydroxybenzoate, 0.1 g chlorobutanol and acetic acid suitabe to obtain a pH of 6.5.
- eye ointment containing a therapeutic dose of anti-IFN gamma VHH was prepared according to the conventional method containing 1.0 g of liquid paraffin and a suitable amount of soft paraffin to obtain a total mixture of 100 g.

2: To obtain anti-inflammatory pharmaceutical applications, topical preparations of the present invention contained at least one anti-IFN gamma VHH and a pharmaceutically acceptable carrier. They were prepared as follows:

- Preparation of Base Cream

The reagents for preparing the base cream are as follows (contents for 100 kg base cream): Dimethyl silicon oil (17 kg), Liquid paraffin (9 kg), Stearic acid (7.5 kg), Cetyl alcohol (1 kg),

Stearyl alcohol (3 kg), Glycerol (20 kg), Ethylparaben (0.1 kg), Peregal A-20 (0.45 kg), Softener SG (0.85 kg), 0.01 M Phosphate Buffer (pH 7.2)(41.1 kg)

The stainless steel tank was placed into a thermostat water bath and heated to 80° C., which took approximately 10 minutes. The liquid was thoroughly mixed. Then, emulsifying and homogenizing equipment was placed into the open stainless steel tank, the mixture was stirred for 20 minutes at 3500 rpm until fully emulsified. The temperature of the thermostat water bath was cooled naturally to room temperature, until the mixture became a semi-solid cream. The mixture was being continually stirred.

- Preparation of Liquid Antibody Mixture

VHH#MP3B1SRA was prepared in accordance with Example 22. The lyophilized antibodies were reconstituted with 0.01 M phosphate buffer (pH 7.2) to a concentration of 2 mg/ml. For 1000 gm of base cream, 45 mg of VHH#MP3B1SRA antibody was added.

Therapeutic VHH-fragments

Example 24: Expression of a VHH-CDR3 fragment of anti-TNFα VHH#3E

The CDR3 region of VHH#3E was amplified by using a sense primer located in the framework 4 region (Forward: CCCCTGGCCCCAGTAGTTATACG) and an anti-sense primer located in the framework 3 region (Reverse: TGTGCAGCAAGAGACGG).

In order to clone the CDR-3 fragment in pAX10, a second round PCR amplification was performed with following primers introducing the required restriction sites:

Reverse primer Sfi1:

GTCCTCGCAACTGCGGCCCAGCCGGCCTGTGCAGCAAGAGACGG

Forward primer Not1:

GTCCTCGCAACTGCGCGGCCGCCCCCTGGCCCCAGTAGTTATACG

The PCR reactions were performed in 50 μl reaction volume using 50pmol of each primer. The reaction conditions for the primary PCR were 11 min at 94 °C, followed by 30/60/120 sec at 94/55/72 °C for 30 cycles, and 5 min at 72°C. All reaction were performed wit 2.5 mM MgCl2 , 200 mM dNTP and 1.25U AmpliTaq God DNA Polymerase (Roche Diagnostics, Brussels, Belgium).

After cleavage with Sfi1 and Not1 the PCR product was cloned in pAX10.

EGFR

Example 25: Immunization

After approval of the Ethical Committee of the Faculty of Veterinary Medicine (University Ghent, Belgium), 4 llamas (024, 025, 026 and 027) were immunized with the tumor antigen epidermal growth factor receptor (EGFR) according to all current animal welfare regulations. To generate an antibody dependent immune response (Table 9), two animals were injected with intact human vulvar squamous carcinoma cells (A431, ATCC CRL 1555), expressing EGFR on its cell surface, while A431 derived membrane extracts were administered to two other llamas (026 and 027). Each animal received seven doses of subcutaneously administered antigens at weekly intervals (Table 9). When immunizing with intact cells, each dose consisted of 10^8 freshly harvested A431 cells. The dose for immunization with membrane extracts consisted of vesicles prepared from 10^8 A431 cells. Vesicles were prepared according to Cohen and colleagues (Cohen S, Ushiro H, Stoscheck C, Chinkers M, 1982. A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J. Biol. Chem. 257:1523-31). Vesicles were stored at -80°C before administration. Two extra injections of eight microgram purified EGFR (Sigma) in an emulsion with the adjuvant Stimune (CEDI Diagnostics B.V., Lelystad, The Netherlands) were administered intramuscularly to llama 025 (Table 9).

Example 26: Evaluation of immune response

At day 0, 28 and 42, 10 ml of (pre-)immune blood was collected and serum was used to evaluate the induction of the immune responses in the 4 animals. A first ELISA was performed to verify whether the animals generated antibodies that recognized A431 epitopes. After coating a tissue-culture treated 96-well plate with gelatin (0.5% in PBS for 10 minutes), the excess of gelatin was removed and A431 cells were grown overnight in the microwells to confluency. Cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Subsequently, the fixative was blocked with 100mM glycine in PBS for 10 minutes, followed by blocking of the wells with a 4% skim milk-PBS solution, again for 10 minutes. Serum dilutions of immunized animals were applied and A431 specific antibodies were detected with a polyclonal anti-llama antiserum developed in rabbit, followed by a secondary goat anti-rabbit horse radish peroxidase (HRP) conjugate (Dako, Denmark). For all

four animals, immunization with intact cells or membrane vesicles resulted in the induction of a significant A431-specific antibody titer (Figure 5).

To verify whether the induced llama antibodies were EGFR specific, antibody titers in serum was evaluated on mouse fibroblasts expressing human EGFR (Her-14) and compared to the parental mouse fibroblasts cell line NIH3T3 clone 2.2 (3T3), similarly performed as described above (Figure 6). Again, the serum titer of antibodies binding to Her-14 was higher compared to the titer for the parental 3T3 cells, indicating that circulating serum antibodies were EGFR specific.

Finally, the serum response in immunized animals was verified on solid-phase coated purified EGFR. Purified EGFR (Sigma) and the irrelevant carcino embryonic antigen (CEA, Scripps), both at 1 μg/ml, were immobilized overnight at 4°C in a 96 well Maxisorp plate (Nunc). Wells were blocked with a casein solution (1% in PBS). After addition of serum dilutions, specifically bound immunoglobulins were detected using a rabbit anti-llama antiserum followed by a goat anti-rabbit alkaline phosphatase conjugate (Sigma), showing that for all animals a significant antibody dependent immune response against EGFR was induced (Figure 7).

Example 27: Cloning of the heavy-chain antibody fragment (VHH) repertoire

Since little is known on the immunoglobulin ontogeny of camelids, B-cell containing tissues of distinct origin and of different time points were collected for each animal (Table 9). After tissue collection, total RNA was isolated according to the procedure described by Chomczynski and Sacchi. (Chomczynski P and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159). The procedure to clone the VHH repertoire is based on a method described in patent application WO 03/054016. cDNA was prepared on total RNA with MMLV Reverse Transcriptase (Invitrogen) using oligo d(T) oligonucleotides (de Haard HJ, van Neer N, Reurs A, Hufton SE, Roovers RC, Henderikx P, de Bruine AP, Arends JW, Hoogenboom HR. 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274:18218-30). The amounts of RNA of the distinct tissues used for cDNA synthesis is listed in Table 10. The cDNA was purified with a phenol/chloroform extraction, followed by an ethanol precipitation and subsequently used as template to amplify the VHH repertoire.

In a first PCR, the repertoire of both conventional (1.6 kb) and heavy chain (1.3 kb) antibody gene segments were amplified using a leader specific primer (ABL002) and ABL010, an oligo

d(T) primer (for a list of primers see Table 13). The resulting DNA fragments were separated by agarose gel electrophoresis. The amplified 1.3 kb fragment, encoding heavy chain antibody segments was purified from the agarose gel and used as template in a nested PCR using a mixture of FR1 primers (ABL037-ABL043) and ABL010. The PCR products were digested with *Sfi*I (introduced in the FR1 primer) and *BstE*II (naturally occurring in FR4). Following gel electrophoresis, the DNA fragment of approximately 400 basepairs was purified from gel and 330 ng of amplified VHH repertoire was ligated into the corresponding restriction sites of one microgram of phagemid pAX004 to obtain a library after electroporation of *Escherichia coli* TG1. pAX004 allows the production of phage particles, expressing the individual VHHs as a fusion protein with the geneIII product. The size of the libraries obtained from the distinct tissues collected from the immunized llamas is described in Table 10. As a quality control, a colony PCR using the M13 reverse and a geneIII primer was performed on 24 randomly picked colonies of each library and the percentage of clones containing an insert of the correct size was calculated (Table 10).

Example 28: Evaluation of the cloned repertoire

In a polyclonal phage ELISA, the specificity of the cloned phage repertoire was evaluated on EGFR and on an irrelevant antigen (TNFα). To generate recombinant virions expressing the VHH repertoire as fusion proteins with the geneIII product, the library was grown at 37°C in 10 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD_{600nm} reached 0.5. M13KO7 phages (10^{12}) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 5 minutes at 4,500 rpm at room temperature. The bacterial pellet was resuspended in 50 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 37°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 4,500 rpm at 4°C and supernatant was used to concentrate the phages. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) for 30 minutes on ice and centrifuged for 20 minutes at 4,500 rpm. The pellet was resuspended in 1 ml PBS. Phages were again PEG precipitated for 10 minutes on ice and centrifuged for 10 minutes at 14,000 rpm and 4°C. The pellet was dissolved in 1 ml PBS. One µg/ml of EGFR or TNFα was immobilized in a 96 well Maxisorp plate (Nunc) and incubated overnight at 4°C. Plates were washed 5 times with PBS/0.05%Tween-20 and wells were blocked with a casein solution (1% in PBS) and phage dilutions were added for 2 hrs at room temperature. Bound

phages were detected using the anti-M13 gpVIII-HRP conjugated monoclonal antibody (Amersham Biosciences) and $ABTS/H_2O_2$ as substrate. Plates were read at 405nm after 15 minutes incubation at room temperature. An example of a phage response from a pool of phages rescued from PBL1 libraries of animals 024 and 025 is depicted in Figure 8.

Example 29: Multiple selection strategies to identify EGFR specific nanobodies

Libraries were rescued by growing the bacteria to logarithmic phase (OD_{600}= 0.5), followed by infection with helper phage to obtain recombinant phages expressing the repertoire of cloned VHHs on tip of the phage as gpIII fusion protein (as described in Example 18). When selecting for EGFR specific antibodies, two distinct selection strategies have been followed.

Selection by epitope specific elution

A first selection strategy was based on the fact that EGFR can be purified by affinity chromatography through ligand elution. Four different elution conditions, applying an excess of molecules that compete for the ligand binding site or overlapping epitope(s) were carried out (Table 11). When selection was performed on A431 or Her-14 cells, unselected recombinant phages were mixed for 20 minutes at 4°C with 6 x 10^6 blood cells (mainly monocytes, T- and B-cells) or 2 x 10^7 3T3s, respectively, to deplete for recombinant phages that recognize common, non EGFR-specific epitopes. Unbound phages were then incubated with $EGFR^+$ selection cells for 2 hours followed by 6 washes with ice-cold PBS. Phages were subsequently eluted with an excess of EGF ligand, mouse monoclonal 2e9 (Defize LH, Moolenaar WH, van der Saag PT, de Laat SW 1986. Dissociation of cellular responses to epidermal growth factor using anti-receptor monoclonal antibodies. EMBO J. 5:1187-92) or EGFR antagonistic antibodies 225 and 528 (Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH 1983. Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. Mol. Biol. Med. 1:511-529). All selection steps were performed at 4°C to avoid receptor mediated phage internalization. Logarithmically grown *E. coli* TG1 was infected with the eluted phages and grown overnight at 37°C on selective medium 2xTY Ap100 and 2% glucose. Cells were scraped and used in a next round of panning whenever required. Two or three rounds of panning were performed to enrich for EGFR specific recombinant phages (Table 11). Whenever purified antigen was used for selection (Table 11), EGFR was immobilized at 1 μg/ml on Maxisorp microtiter plates.

Selection for internalizing VHH fragments

A second selection strategy was based on the observation that after binding of the ligand to the receptor, EGFR mediated cell signaling can be downregulated by the mechanism of receptor internalization. To identify recombinant phages that are able to internalize through cell surface molecules, the protocol described by Poul and colleagues (Poul MA, Becerril B, Nielsen UB, Morisson P, Marks JD. 2000. Selection of tumor-specific internalizing human antibodies from phage libraries. J. Mol. Biol. 301:1149-61.) was followed. Unselected recombinant phages were added to approximately 2 x 10^7 mouse fibroblast 3T3s for 30 minutes at 4°C in ice cold binding medium (bicarbonate buffered DMEM; 10% FCS (featal calf serum); 25mM Hepes), supplemented with 2% skim milk to deplete for non-specific VHHs. Unbound phages were subsequently incubated with pre-cooled $EGFR^+$ selection cells (Her-14 or A431) in binding medium for 1.5 hours at 4°C, followed by six washes with ice-cold PBS to remove non-bound phages. Cells were covered with pre-warmed binding medium and immediately transferred to 37°C for 20 minutes, to allow internalization. Subsequently, cells were cooled down to 4°C and were stripped with mild acid (500mM NaCl; 100mM glycine pH2.5) incubations during 10 minutes to remove surface bound recombinant phages. Cells were released from extracellular matrix by trypsinization. Resuspended cells were then lyzed during 4 minutes with 100mM TEA at 4°C to release internalized phages. Logarithmically grown *E. coli* TG1 was infected with the eluted phages and grown overnight at 37°C on selective medium (2xTY Ap100 with 2% glucose). The libraries used for a single round of selection on A431 and in parallel on Her-14 are summarized in Table 12.

Example 30: Characterization of EGFR specific nanobodies

To verify EGFR specificity of individual clones after the epitope specific elution procedure of panning, a phage ELISA was performed on individual clones. 47 randomly picked clones for each selection procedure (1, 2, 3, 4, Ia and IIIa; Table 11) were grown to logarithmic phase (OD_{600}= 0.5), followed by infection with helper phage to obtain recombinant phages as described in Example 18. A phage ELISA was performed both on solid-phase coated EGFR (comparing to non-coated well) as on gelatin coated Her-14 cells (comparing to 3T3). The presence of EGFR specific VHH was verified by using approximately 10^9 recombinant phage particles of each clone before detection with an anti-M13 gpVIII-HRP conjugated monoclonal antibody. With clones that scored positive in phage ELISA on cells and/or on solid-phase immobilized EGFR (Table 11), a *Hin*fI fingerprint analysis was performed (data not shown).

The nucleotide sequence was determined for a representative clone of each distinct fingerprint, resulting in 5, 8, 3, 4, 7, and 4 different sequences for conditions, 1, Ia, 2, IIIa, 3 and 4, respectively. Amino acid sequence alignment of these 31 binders (Figure 9) indicated that 20 of them were unique (listed in Table 14 SEQ ID Nos 23 to 42). The EGFR specificity of the 20 unique clones in phage ELISA (both on cells and on solid-phase coated EGFR) is shown in Figure 10.

For the selection according to the internalization protocol, a phage ELISA on cells with a total of 84 individual clones was performed, similarly as for the clones identified by the epitope specific elution selection procedure. After *HinfI* fingerprint analysis, nucleotide sequence determination and amino acid sequence alignment to the above described panel of 20 unique binders (data not shown), 2 new anti-EGFR clones, EGFR-B11 and clone EGFR-F11, were identified (Table 14 SEQ ID NOs: 43 to 44). The EGFR specificity of both clones in phage ELISA on cells is shown in Figure 10, panel A.

Example 31: EGF receptor mediated internalization of nanobodies.

Her-14 and 3T3 cells were grown overnight on glass cover slips, washed with binding medium (see Example 19) and cooled down to 4 °C for 20 minutes. Phages were prepared of nanobody EGFR-IIIa42 as described in Example 18 and approximately 10^{12} recombinant virions, diluted in binding medium supplemented with 2% skim milk, were added to the ice cold cells for 1 hour at 4 °C. Cells were washed once with ice cold PBS to remove non bound phages. Subsequently, the cells were shifted to 37°C for 20 minutes to allow phage internalization and again cooled down to 4°C. Cells were washed twice with PBS. Following, cell surface bound phages were removed by two acid washes with stripping buffer (150 mM NaCl, 125 mM HAc) for seven minutes at room temperature. After two washes with PBS, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature, and again washed twice with PBS. Fixed cells were then permeabilized in 0.2% Triton X-100 in PBS for 5 minutes at room temperature, followed by two washes with PBS and remaining fixative was blocked with 100mM glycin in PBS for 10 minutes at room temperature. Cells were washed with PBS-0.5% (w/v) gelatin and internalized phage was visualized by staining with anti-M13 gpVIII-FITC (Amersham Biosciences) followed by an anti-mouse FITC labeled monoclonal antibody and subsequent visualization by fluorescence microscopy. Figure 11 shows that EGFRIIIa42 is able to internalize Her-14 (panel A) but not 3T3 cells (panel B).

Subsequently, FACS analysis demonstrated that nanobody EGFR-IIIa42 is able to bind both A431 and Her-14, but not 3T3 (data not shown).

To demonstrate the effect of EGF receptor specific nanobodies on receptor signalling, cells were seeded at 100,000 cells per well in 12-well tissue culture plates in medium (DMEM) containing 10% (v/v) serum. After 8 hours, cells were washed once with medium (DMEM) containing low (0.5% v/v) serum and serum-starved overnight in the same medium. The day of the assay, medium was refreshed with binding medium (DMEM/ 0.5% FCS / 25mM Hepes and 2% skim milk) and when appropriate, ligand or nanobody (mono- or bivalent) was added at 37°C. After 15 minutes, cells were quickly cooled down on ice and washed twice with ice-cold PBS (10mM Na-phosphate; 150mM NaCl, pH 7.4). Total cell lysates were prepared by scraping the cells off the plate in 50µl protein sample buffer. Proteins were size-separated on 6% (w/v) poly-acrylamide gels (20µl loaded per gel on two parallel gels) and blotted to PVDF membrane (Roche). Blots were stained for total amount of EGFR with a rabbit polyclonal antiserum to the receptor (Santa Cruz) and for phosphorylated receptor using a monoclonal anti phospho-tyrosine antibody (PY-20; Transduction Labs), followed by an appropriate in donkey developped and peroxidase conjugated secondary antibody (anti-rabbit or anti-mouse). The detection was performed by enhanced chemoluminiscence using Western Lightning™ substrate (Perkin Elmer Life Sciences). Surprisingly, anti-EGFR-IIIa42 nanobody did not activate $EGFR^+$ cells deprived from EGF, indicated by the lack of receptor Tyr kinase phosphorylation (Fig 11, panel C). The positive control, in which EGF was added in two concentrations to the cells, clearly induced phosphorylation of the receptor and thus induces activation of the cells.

PDK1

Example 32 (1): Immunisation of llamas

2 llamas are immunised with a cocktail of recombinant EGF receptor and with PDK1. The lamas are boosted with a cell line overexpressing the EGF receptor. The immunization schemes are summarised in Table 15.

Example 33: Repertoire cloning

Different sources for RNA extraction are used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) are isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node are used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire is amplified using two hinge-specific primers: AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer: GAGGTBCARCTGCAGGASTCYGG. Fragments are digested with PstI and NotI and cloned into a phagemid vector. The repertoire is transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 μg/ml ampicillin and 2% glucose. Colonies are screened for the presence of insert by PCR with vector specific primers.

Example 34: Rescue of the library, phage preparation

Libraries are grown at 37°C in 60 ml 2xTY medium containing 2% glucose, and 100 μg/ml ampicillin, until the OD600nm reached 0.5. M13KO7 phages (1012) are added and the mixture is incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells are centrifuged for 10 minutes at 4500 rpm at room temperature. The bacterial pellet is resuspended in 300 ml of 2xTY medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin, and incubated overnight at 30°C with vigorously shaking at 250 rpm. The overnight cultures are centrifuged for 15 minutes at 10.000 rpm at 4°C. Phages are PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) and centrifuged for 30 minutes at 10.000 rpm. The pellet is resuspended in 20 ml PBS. Phages are again PEG precipitated and centrifuged for 30 minutes at 20,000 rpm and 4°C. The pellet is dissolved in 5 ml PBS.

Phages are titrated by infection of TG1 cells at OD600nm= 0.5 and plating on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The number of transformants indicates the number of phages (pfu). The phages are stored at –80°C with 15% glycerol.

Example 35: Selection

Immunotubes are coated with 2 µg/ml EGFR, 2 µg/ml PDK1 or with PBS containing 1% casein. After overnight incubation at 4°C, the tubes are blocked with PBS containing 1% casein, for 3 hours at RT. 200 µl phages of the three libraries of llama 005 and of the three libraries of llama006 are pooled and added to the immunotubes with a final volume of 2 ml in PBS for EGFR and in 50 mM Tris HCl (pH 7.4), 150 mM KCl, 1.0 mM DTT, 1 mM MgCl2 and 0.3 mg/ml BSA for PDK1.

After 2 hours incubation at RT, the immunotubes are washed 10x with PBS-Tween and 10x with PBS. Bound phages are eluted with 2 ml 0.2 M glycin buffer pH= 2.4. Eluted phages are allowed to infect exponentially growing TG1 cells, and are then plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Examples of results which might be obtained from the panning are presented in Tables 16 and 17.

Example 36: Screening

A microtiter plate is coated with 2 µg/ml EGFR or 2 µg/ml PDK1, overnight at 4°C. Plates are blocked for two hours at room temperature with 300 µl 1% casein in PBS. The plates are washed three times with PBS-Tween. Periplasmic extracts are prepared from single colonies and applied to the wells of the microtiter plate. Plates are washed six times with PBS-Tween, after which binding of VHH is detected by incubation with mouse anti-Histidine mAB 1/1000 in PBS for 1 hour at RT followed by anti-mouse-alkaline phosphatase conjugate 1/2000 in PBS, also for 1 hour at RT. Staining is performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in 1M diethanolamine, 1mM Mg_2SO_4, pH9.8) and the signals are measured after 30 minutes at 405 nm. An example of the expected number of positive clones versus the number of clones tested in ELISA for each selection is presented in Table 18.

Example 37: Screen for internalised VHH

Individual clones specific for the EGFR are amplified by PCR and cloned in a phage engineered to package the green fluorescent protein reporter gene driven by the CMV promoter (Poul MA et al, J Mol Biol, 1999, 288: 203-211). Phages are prepared and incubated with tumor cells (A431) overexpressing EGFR. Phages that endergo EGFR mediated endocytosis are be measured by GFP expression. 1 VHH (EGFR-21) would be expected to show a very high expression of GFP and would be used for further analysis. In another approach internalised phage is stained with anti-phage antibodies (poly- or monoclonal) after permeabilization of cells by treatment with cold methanol as described by Larocca and colleagues (Larocco et al, Molecular Therapy, 2001, 3: 476 – 484) and by Poul and colleagues (Poul MA et al, J Mol Biol, 1999, 288: 203-211).

Example 38: Screen for VHH inhibiting PDK1-Akt interaction

PDK1 is coated in a microtiter plate as described above and after blocking the plates, the wells are incubated with 100 µg/ml Akt for one hour at RT. Then (without washing) 100 µl periplasmic extract is added to those wells and VHH binding is measured as described above. VHH that are not able to bind to PDK1 would be scored as inhibitors for the interaction between PDK1 and Akt. The expected number of inhibiting VHH versus the number of VHH tested in inhibition ELISA is summarized in Table 19.

Example 39: Making a bispecific construct

A bispecific construct is prepared (Conrath et al, J Biol Chem, 2001, 276: 7346-7350) of EGFR-21 and 5 different strong inhibiting VHHs (PD-1, PD-7, PD-32, PD-33 and PD-72) for PDK1. Protein is prepared and purified to homogeneity for the 5 bispecific constructs and shown to be stable by western blot analysis.

Example 40: Endocytosis and lysis of tumor cells

Bispecific constructs are incubated with tumor cells (A431) overexpressing EGFR. All constructs that successfully endocytosed would be shown by confocal microscopy. One of the constructs, EGFR-21-PD-32, would be expected to able to inhibit cell growth and finally lead to cell death.

Example 41: Calculation of homologies between anti-target-single domain antibodies of the invention

The degree of amino acid sequence homology between anti-target single domain antibodies of the invention was calculated using the Bioedit Sequence Alignment Editor. The calculations indicate the proportion of identical residues between all of the sequences as they are aligned by ClustalW. (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research, submitted, June 1994). Table 20 indicates the fraction homology between anti-TNF-alpha VHHs of the invention. Table 21 indicates the percentage homology between anti-IFN-gamma VHHs of the invention.

Example 42: Construction of a bispecific constructs containing a VHH-CDR3 fragment fused to an anti-serum albumin VHH

A functional portion, the CDR3 region of MP2F6SR, was amplified by using a sense primer located in the framework 4 region (F6 CRD3 Forward:CTGGCCCCAGAAGTCATACC) and an anti-sense primer located in the framework 3 region (F6 CDR3 Reverse primer:TGTGCATGTGCAGCAAACC).

In order to fuse the CDR-3 fragment with the anti-serum albumin VHH MSA-21, a second round PCR amplification was performed with following primers:

F6 CDR3 Reverse primer Sfi1:

GTCCTCGCAACTGCGGCCCAGCCGGCCTGTGCATGTGCAGCAAACC

F6 CDR3 Forward primer Not1:

GTCCTCGCAACTGCGCGGCCGCCTGGCCCCAGAAGTCATACC

The PCR reactions were performed in 50 ml reaction volume using 50pmol of each primer. The reaction conditions for the primary PCR were 11 min at 94 °C, followed by 30/60/120 sec at 94/55/72 °C for 30 cycles, and 5 min at 72°C. All reaction were performed wit 2.5 mM MgCl2 , 200 mM dNTP and 1.25U AmpliTaq God DNA Polymerase (Roche Diagnostics, Brussels, Belgium).

After cleavage of the VHH gene of MSA clones with restriction enzymes Pst1/BstEII the digested products were cloned in pAX11 to obtain clones with a VHH at the C-terminus of the

multicloning site. The clones were examined by PCR using vector based primers. From clones yielding a 650 bp product, DNA was prepared and used as acceptor vector to clone the CDR3 of MP2F6SR, after cleavage of the PCR product with restriction enzymes Sfi1/Not1 to allow N-terminal expression of CDR3 in fusion with a MSA VHH.

TABLES

Day	Llama 2	Llama 4
0	100 µg	100 µg
7	100 µg	
14	50 µg	
21	50 µg	100 µg
28	50 µg	
35	50 µg	
42		50 µg
70		50 µg

Table 1: Immunization scheme as described in Example 1

	#days after last injection	Source RNA	Size of the library	% insert
Llama002	4	Lymph	1.3×10^7	89
	4	PBL	1.9×10^7	95
	10	PBL	1.1×10^9	70
Llama004	4	PBL	1.7×10^8	96
	4	Lymph	4.9×10^7	>95
	10	PBL	2.2×10^6	>95

Table 2: Presence of insert by PCR with vector specific primers as described in Example 1

	5 µg/ ml	0.5 µg/ ml	0 µg/ ml (blanco)
Llama 2 (pool PBL day4, PBLday10, lymph node day4)	1.4 10^{6}	2.7 10^{5}	1.5 10^{4}
Enrichment compared to blanco	*400 x*	*18 x*	
Llama 4 (pool PBL day4, PBLday10, lymph node day4)	3.3 10^{6}	4.5 10^{5}	7.2 10^{4}
Enrichment compared to blanco	*140 x*	*6.25 x*	

Table 3: First selection as described in Example 1

	1 µg/ ml	**1 µg/ ml**	**0 µg/ ml**	**0 µg/ ml**
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme
Llama 2 (selection 5 µg/ ml IgE: 400 x enrichment)	1.2 10^{8}	1.2 10^{8}	6 10^{3}	3 10^{3}
Enrichment compared to lysozyme elution	No enrichment			2x
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	1.3 10^{8}	2 10^{7}	3 10^{3}	3 10^{3}
Enrichment compared to lysozyme elution	6.5 x		No enrichment	

Table 4: Second selection using the rescued phages from the first selection as described in Example 1

	2 nM IgE	**2 nM IgE**	**0 nM IgE**	**0 nM IgE**
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme
Llama 2 (selection 5 µg/ ml IgE:	1.5 10^{8}	1.5 10^{7}	3 10^{5}	3 10^{3}

400 x enrichment)				
Enrichment compared to lysozyme elution	10 x			
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	$3.3\ 10^7$	$2.2\ 10^7$	$3\ 10^3$	$6\ 10^3$
Enrichment compared to lysozyme elution	1.5 x			

Table 5: Second round selection using neutravidine coated tubes as described in Example 1

	Selection with 5 µg/ml	**Selection with 0.5 µg/ml**
Llama 002	39/47	21/47
Llama 004	45/47	46/47

Table 6: Number of clones that score positive for binding to both human IgE and chimeric IgE versus the number of clones tested in ELISA as described in Example 1

Group	**Animals**	**Description**	**Schedule**
1	8	negative control 1 ip	daily 100 µl PBS i.p. +
2	8	negative control 2 rectal	every other day 100 µl PBS rectal for 2 weeks
3	8	negative control 3 intragastric	daily 100 µl PBS intragastric for 14 consecutive days
4	8	positive control 1 dexamethasone	5 µg i.p. for 7 consecutive days
5	8	positive control 2 IL10 expressing l.lactis	applied orally once per day for 14 consecutive days
6	8	bivalent VHH 3F intra-gastric	daily 100 µg bivalent VHH $3F_2$ intragastric on 14 consecutive days
7	8	bivalent VHH 3F i.p.	daily 100 µg bivalent VHH 3F i.p. for 14 consecutive days
8	8	bivalent VHH 3F rectally	100 µg bivalent VHH 3F rectally in 100 µl PBS every other day for two weeks

Table 7: Treatment schedule

Animal	Antigen	Source	Titer	% Insert
Llama 5	Human MMP-12	PBL time 1	2.1 10^8	94%
Llama 5	Human MMP-12	PBL time 2	7.5 10^6	92%
Llama 5	Human MMP-12	Lymph node	7.8 10^8	100%

Table 8: Overview of the libraries, their diversity and % insert derived from different llama's and tissues as described in Example 7 and 8

Day	Llama 024	Llama 025	Llama 026	Llama 027
0	intact cells	intact cells	vesicles	vesicles
7	intact cells	intact cells	vesicles	vesicles
14	intact cells	intact cells	vesicles	vesicles
21	intact cells	intact cells	vesicles	vesicles
28	intact cells	intact cells	vesicles	vesicles
35	intact cells	intact cells	vesicles	vesicles
42	intact cells	intact cells	vesicles	vesicles
46	150 ml blood sample (PBL1)	150 ml blood sample (PBL1)	150 ml blood sample (PBL1) lymph node	150 ml blood sample (PBL1) lymph node
47	lymph node spleen bone marrow			
49		purified EGFR	150 ml blood sample (PBL2)	150 ml blood sample (PBL2)
55		purified EGFR		
59		150 ml blood sample (PBL2)		
60		lymph node spleen bone marrow		

Table 9: Immunization schedule and tissue collections

Animal	Tissue	RNA (μg)	Size (x 10^8)	% Insert
Llama 024	PBL1	200	0.25	83
Llama 024	Lymph node ileum	40	2.3	78
Llama 024	Lymph node bow	150	0.17	100
Llama 024	Bone marrow	97	1.5	83
Llama 024	Spleen	160	0.16	95
Llama 025	PBL1	200	0.06	95
Llama 025	Lymph node (ileum + bow)	200	0.8	96
Llama 025	Bone marrow	200	0.045	88
Llama 025	Spleen	200	2	86
Llama 025	PBL2	200	0.13	83
Llama 026	PBL1 + lymph node	100 + 200	2.46	85
Llama 027	PBL1 + lymph node	100 + 200	1.08	92

Table 10: Overview of constructed libraries

Elution condition	Elution molecule	Selection: antigen format			ΦELISA Her-14	ΦELISA EGFR	Binder families
		Round I	Round II	Round III			
1 Ia	EGF	A431	Her-14 EGFR	-	1/47 5/47	24/47 23/47	6 8
2 IIIa	2e9	A431	Her-14 EGFR	-	2/47 11/47	32/47 32/47	5 4
3	225	A431	A431	Her-14 EGFR	8/47 20/47	28/47 31/47	5
4	528	A431	A431	Her-14 EGFR	16/47 22/47	10/47 29/47	5

Table 11: Overview of epitope specific elution selection procedure

Library	Selection cells	Selected antibody fragment
Pool lymph node, bone marrow, spleen and PBL1 (024+025)	Her-14	A2
Pool bone marrow (024+025)	A431	A4, A9, B11
Pool PBL1 (024+025)	A431	F11

Table 12: Overview of 'internalization' selection procedure

Name	Sequence 5' – 3'
ABL002	GGCTGAGCTCGGTGGTCCTGGCT
ABL010	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTT
ABL037	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGA GTCTGG
ABL038	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGATGTGCAGCTGGTGGA GTCTGG
ABL039	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGCGGTGCAGCTGGTGGA GTCTGG
ABL040	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGCCGTGCAGCTGGTGGA TTCTGG
ABL041	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGGA GTCTGG
ABL042	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCCAGGTACAGCTGGTGGA GTCTGG
ABL043	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCCAGGTAAAGCTGGAGGA GTCTGG
geneIII	CCACAGACAGCCCTCATAG
M13 rev	GGATAACAATTTCACACAGG

Table 13: Primer sequences

SEQ ID NO	NAME	SEQUENCE
		Anti-IgE VHH
1	VHH#2C3	QVQLQDSGGGLVQPGGSLRLSCRASGRIFRINAMGWYRQAPGKQRELVATI TSTGSTNFADSVKGRFTIYRDGAKRTVDLRLNSLKPEDTAVYFCNADVREY DLGPWRQYWGQGTQVTVSS
2	VHH#4G12	QVQLQESGGGVVQPGGSLRLSCSVSGTSISNRVMAWFRQAPGKQRDFVAYI TSAVNTDYADFVKGRFTISRDNAQNMVHLQMNSLKPEDTAVYYCNVLKDTW FRTPYDYYWGQGTQVTVSS
3	VHH#2C1	QVQLQESGGGLVQPGDSLRLSCVVSGRTLSYSSLAWFRQAPGKERDFVAAL SLTTYY ADSVKGRFTISRDNAKNTVYLQMNSLKPDDTADYFCATARTRTDYAPLLSA ASTYDAWGQGTQVTVSL
4	VHH#2H3	QVQLQESGGGLVQAGGSLRLSCAASGRSSRYYAMGWFRQGPGKEREFVAAV NWNGDSTYYADSVKGRFTISRGNAENTAYLQMNSLVPEDTAVYYCAMRMNA GLGYSAASYQYWGQGTQVTVSL
5	VHH#2D12	QVQLQESGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAI DWSGRRITYTDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAADRTY SYSSTGYYYWGQGTQVTVSS
6	VHH#2G4	QVQLQDSGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREFVGAI DWSGRRITYTDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVYYCAADRTY SYSSTGYYYWGQGTQVTVSS
7	VHH#4C5	QVQLQESGGGLVQAGGSLRLSCAASGRTLSSYTMAWFRQAPGKEREFVASI SSSGISTYYADSVKGRFTISRDIAKNTVYLQMNSLKPEDTAVYYCAAKYRY YSTLYTKSGEYDYWGQGTQVTVSS
8	VHH#4A2	QVQLQDSGGGLVQAGGSLRLSCEASGRTISSYAMAWFRQAPGKEREFVASI SSSGVSKHYADSVKGRFTISNDKVKNTVYLQMNSLKPEDTAVYFCAAKYRY YSSYYTKSGDYDYWGQGTQVTVSS
9	VHH#2D4	QVQLQESGGGLVQAGGSLRLSCAASGLTFSTYAMGWFRQAPGKEREFVAAV SYSGSYYADSVKGRFTISRDNAKNTVYLQMASLKPEDTAVYYCAARNRGYS TYAGVYDYWGQGTQVTVSS
10	VHH#2B6	QVQLQDSGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASI TWIGGGTYYADSVKGRFTISRDHAGNTVYLQMNTLKPDDTAVYYCALDRRS STYYLMKGEYDYRGRGTQVTVSS
11	VHH#2H11	QVQLQESGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASI TWTGTGTYYADSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVDRRS STYYLMKGEYDYRGRGTQVTVSS
		Anti-TNF alpha VHH
12	VHH#3E-His tag	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPKEREF VARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAA RDGIPTSRSVESYNYWGQGTQVTVSSAAAEQKLISEEDLNGAAHHHHHH
13	VHH#3F	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAV SWSGGTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQ KYNWASASYNVWGQGTQVTVSS

14	VHH#3F/ VHH#3F	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAV SWSGGTTVYADSVLGRFEISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQ KYNWASASYNVWGQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQAGGS LRLSCAASGGTFSSIIMAWFRQAPGKEREFVGAVSWSGGTTVYADSVLGRF EISRDSARKSVYLQMNSLKPEDTAVYYCAARPYQKYNWASASYNVWGQGTQ VTVSS
		Human MMP-12 specific VHH
15	MMP-12 P1-1	QVQLQESGGGLVQPGGSLRLSCVASGFTFSDYPMAWVRQAPGKGLEWISVI NSGGVNTSYAASVKGRFTISRDNAKNTLFLQMNSLKPEDTAVYYCAKYSLK NEQYWRGQGTQVTVSS
16	MMP-12 P1-3	QVQLQESGGGLVQPGGSLRLSCAASGSIFSIDGMGWYRQAPGKQRERKQRE LVAAITSGGSTKYADSVKGRFTISRDNANDTVYLQMNTLKPEDTAVYYCNA VLLRRGIVYDYWGQGKQVTVSS
17	MMP-12 P1-7	QVQLQESGGGSVKAGGSLRLSCAASGSIFSIDGMGWYRQAPGKQRERKQRE LVAAITSGGSTKYADSVKGRFTISRDNANDTVYLQMNTLKPEDTAVYYCNA VLLRRGIVYDYWGQGKQVTVSS
18	MMP-12 P1-26	QVQLQESGGGLVRAGGSLRLSCVASGRTLSKYRMGWFRQFPGKERELVAEI EWKSSSTWYRDSVKGRFTISRDNAKNTVYLRMNSLKPEDTAVYYCAAATLG EPLVKYTYWGQGTQVTVSS
19	MMP-12 P1-33	QVQLQESGGGLVQPGGSLRLSCAASGSIFSIDGMGWYRQAPGKQRERKQRE LVAAITSGGSTKYADSVKGRFTISRDNANDTVYLQMNTLKPEDTAVYYCNA VLLRRGIVYDYWGQGKQVTVSS
20	MMP-12 P1-41	QVQLQDSGGGLVRTGDSLRLSCVVFGGTISTYAMGWFRRAPGKEREFVAAI DASGGFTEYADSVRGRFRIARDNPLSAVYLQMNSLKPEDTAFYYCAADKDR DTVVRFTTTPNEYDYWGQGTQVTVSS
21	MMP-12 P1-44	QVQLQESGGGLVQPGGSLRLSCAASGFTFNNHWLYWVRQAQGKGLEWVSAI NPGGSTVYLDSVKGRFTISRGNTKNTLYLQMNSLKSEDTAVYYCTKAMAWA TDWDEYDLWGQGTQVTVSS
22	MMP-12 P5-29	QVQLQESGGGLVQAGGSLRLSCAASGRTFTVYTTGWFRQAPGKEREFVAAI DWSGSSTYYTDSVKGRFTISRDNTKNTVYLQMNSLKPEDTAVYYCAARDAI VGVTDTSGYRYWGQGTQVTVSS
		Anti-EGFR VHH
23	EGFR-1.4	EVQLVESGGGLVQAGGSLRLSCAASGRTFSNYVMGWFRQAPGKERDFVVGI GRSGGDNTYYADSVKGRFTISWDNAKNTMYLQMNSLKPEDTAVYYCAASTY SRDTIFTKWANYNYWGQGTQVTVSS
24	EGFR-1.9	QVQLQESGGGLVKAGGSLRLSCAASGRTFSSYVMGWFRQAPGKEREFVGAI HWSGGRTYYADSVKGRFTISSDNAKNTLYLQMNSLKPEDTAVYYCAASRII YSYVNYVNPGEYDYWGQGTQVTVSS
25	EGFR-1.33	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSHYMSWFRQAPGKEREFVAAI TSSSRTYYTESVKGRFTISRDNAKNTVYLQMNSLKSEDTAVYYCAADRTFY GSTWSKYDYRGQGTQVTVSS
26	EGFR-1.34	QVQLQESGGGLVQAGGSLRLSCAASGRTFSKYAMGWFRQAPGKEREFVSAI SWSDGSTYYADSVKGRFTISRDNAKNTVYLQVNSLKPEDTAVYYCAATYLV DVWAVHVPIRPYEYDYWGQGTQVTVSS
27	EGFR-1.38	QVQLQDSGGGLVQAGDSLRLSCAASGRSFGGYAMGWFRQAPGKEREFVAAI SWSGGSTYYADSLKGRFTISRDNAKNTVYLQMNSLKPEDTALYYCAAGLRP SPNYNHERSYDYWGQGTQVTVSS
28	EGFR-Ia1	QVQLQESGGGLVQAGGSLLLSCAASGRTFSSYAMGWFRQAPGKEREFVAAI NWSGGSTSYADSVKGRFTISRDNTKNTVYLQMNSLKPEDTAAFYCAATYNP

		YSRDHYFPRMTTEYDYWGQGTQVTVSS
29	EGFR-Ia7	QVQLQESGGRLVQTGGSLRLSCAASGGTFGTYALGWFRQAPGKEREFVAAI SRFGSTYYADSVKGRFTISRDNANNTVYLEMNSLKPEDTAVYYCAAREGVA LGLRNDANYWGQGTQVTVSS
30	EGFR-Ia15	QVQLQDSGGGLVQAGGSLRLSCAASGGTFSSYAMGWFRQAPGKEREFVAAI GLNTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAARTSGVVG GTPKRYDYWGQGTQVTVSS
31	EGFR-IIIa42	EVQLVESGGGSVQAGGSLKLSCAASGRSFSTYAMGWFRQAPGQDREFVATI SWTDSTDYADSVKGRFTISRDNAKNTGYLQMNSLKPEDTAVYYCAADRWAS SRRNVDYDYWGQGTQVTVSS
32	EGFR-2.6	QVQLQESGGGLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFVAAI NWGGGNTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAASEWG GSDYDHDYDYWGQGTQVTVSS
33	EGFR-2.20	EVQLVESGGGLVQAGGSLRLSCAASGRSFSSYAMAWFRQAPGKEREFVAAI SWGGGSTYYAVSVKGRFTISRDNAKNTVYLQMNSLKPEDTARYYCAADETF HSSAYGEYEYWGQGTQVTVSS
34	EGFR-IIIa5	EVQLVESGGGLVQAGGSLRLSCTASGRTFSSYAMGWFRQTPGKEREFVAAI TSSGGSTYYADSVKGRFTISRDNAKSTMYLQMDSLMLDDTSVYYCAADSSR PQYSDSALRRILSLSNSYPYWGQGTQVTVSS
35	EGFR-3.18	EVQLVESGGGLVQPGGSLRLSCVASGFTFADYAMSWVRQAPGKGLQWVSSI SYNGDTTYYAESMKDRFTISRDNAKNTLYLQMNSLKSEDTAVYYCASSGSY YPGHFESWGQGTQVTVSS
36	EGFR-3.32	QVQLQESGGGLVQAGGSLRLSCAASGRTFSGYAMGWFRQAPGEEREFVAAI SWRGTSTYYGDSAKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGSHS DYAPDYDYWGQGTQVTVSS
37	EGFR-3.34	QVQLQESGGGLVQAGGSLRLSCAASGRTFSSYAIGWFRQAPGKEREFVAAI SWGGSNTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAGEVS NSDYAYEYDYWGQGTQVTVSS
38	EGFR-3.39	QVQLQESGGGLVQTGGSLRLSCAASGRYIMGWFRQAPGKEREFVAGISRSG ASTAYADSVKDRFTISRDSALNTVYLQMNSLKAEDTAVYFCAAALAIRLGI PRGETEYEYWGQGTQVTVSS
39	EGFR-3.40	QVKLEESGGGLVQAGGSLRLSCSASGLTFSNYAMAWFRQAPGKEREFVATI SQRGGMRHYLDSVKDRFTISRDNAKNTVYLQMNSLKPDDTAVYYCAADLMY GVDRRYDYWGRGTQVTVSS
40	EGFR-4.11	QVKLEESGGGLVQAGDSLRLSCAASGRSFSSITMGWFRQAPGKERQFVSAI NSNGNRYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAVQAYS SSSDYYSQEGAYDYWGQGTQVTVSS
41	EGFR-4.21	EVQLVESGGGLVQAGGSLRLSCAVSGRTFSSMGWFRQAPGKEREFVATINL SGDRTDYADSVKGRFTISRDNPKNTVYLQMDSLEPEDSAVYYCAGTSLYPS NLRYYTLPGTYADWGQGTQVTVSS
42	EGFR-4.22	QVKLEESGGGLVQAGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVARI TGTGTGITGAVSTNYADSVKGRFTISRDNARNTVYLQMNSLKPEDTAVYYC AADRSRTIVVPDYWGQGTQVTVSS
43	EGFR-B11	QVQLQDSGGGLVQAGGSLRLSCAASRFSSAQYAIGWFRQAPGKEREGVSYI TFSGGPTGYADSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCAARPYT RPGSMWVSSLYDNWGQGTQVTVSS
44	EGFR-F11	QVQLQESGGRLVQAGGSLRLSCAASEHTFRGYAIGWFRQAPGKEREFVSSI TYDGTLTNYADSVTGRFTISRDNAKNTVYLQMNSLKPEDTAVYVCAAGYSY RYTTLNQYDSWGQGTQVTVSS

		Anti - human IFN gamma VHH
45	MP3D2SRA	QVQLQDSGGGTVQAGGSLRLSCAASGRTFSDYAVGWFRQAPGKEREFVARILWTGASRSYANSVDGRFTVSTDNAKNTVYLQMNSLKPEDTAIYYCAALPSNIITTDYLRVYYWGQGTQVTVSS
46	MP3A3SR	QVQLQDSGGGTVQAGGSLRLSCAASGRTFSNYAVGWFRQAPGKEREFVARIKWSGGSRSYANSVDGRFTVSTDNAKNTVYLQMNSLKPEDTAIYYCA?LPSNIITTDYLRVYYWGQGTQVTVSS
47	MP3C5SR	QVQLQESGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWYRQAPGKQRELVAGILTSGATSYAESVKGRFTISRDNAKNTVYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
48	MP3C1SR	QVQLQDSGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWYRQAPGKQRELVAGILSSGATVYAESVKGRFTISRDNAKNTVYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
49	MP3G8SR	QVQLQESGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWYRQAPGKQRELVAGILSSGATAYAESVKGRFTISRDNAKNTVYLQMNSLSPEDTAEYYCNTYPTWVLSWGQGTQVTVSS
50	MP3D2BR	QVQLQESGGGLVQPGESLRLSCAASRGIFRFNAGGWYRQAPGKQRELVAFIGVDNTTRYIDSVKGRFTISRDNAKTTVYLQMNSLQPEDTAVYYCNKVPYIDWGQGTQVTVSS
51	MP3H6SRA	QVQLQESGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQAPGKEREFVAGISWNGGSIYYTSSVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGTQVTVSS
52	MP3B4SRA	QVQLQESGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQAPGKEREFVAGISWNGGSIYYTSSVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGTQVTVSS
53	MP4E4BR	QVQLQESGGGLVQAGGSLRLSCAASGRTFSIYNMGWFRQAPGKEREFVAAISWNGGSIYYTSSVEGRFTISRDNAINTVYLQMNSLKPEDTGVYYCASKGRPYGVPSPRQGEYDYWGQGTQVTVSS
54	MP4H8SR	QVQLQESGGGLVQAGGSLRLSCAASGRTFNIYNMGWFRQAPGKERDFVAAISWNGGSIYYTSSVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYGVPSPRQGDYDYWGQGTQVTVSS
55	MP2F6SR	QVKLEESGGGLVQAGGSLRLSCAASGRTFNNYNMGWFRQAPGKEREFVAAISWNGGSTYYDDSVKGRFTISRDNANNLVYLQMNSLNFEDTAVYYCACAANPYGIPQYRENRYDFWGQGTQVTVSS
56	MP3D1BR	QVQLQESGGGLVQAGGSLRLSCAASGRTFDNYNMGWFRQAPGKEREFVAAISWNGGSTYYDDSVKGRFTISRDNFQKLVYLQMNSLKLEDTAVYYCACAANPYGIPQYRENRYDFWGQGTQVTVSS
57	MP2B5BR	QVQLVESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQAPGKEREFLASVTWGFGSTSYADSVKGRFTISRDKAKDTVYLQMNTLEPDDTSVYYCASSPRYCAGYRCYVTASEFDSWGQGTQVTVSS
58	MP2C1BR	QVKLEESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQAPGKEREFLASVSWGFGSTYYADSVKGRFTISRDTAKDTVYLQMNTLEPDDTSVYYCASSPRYCAGYRCYATASEFDSWGQGTQVTVSS
59	MP4A12SR	QVQLQESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQAPGKEREFLASVTWGFGSTYYADSVKGRFTISRDKAKDTVYLQMNTLEPDDTSAYYCASSPRYCAGYRCYVTASEFDSWGPGTQVTVSS
60	MP3F4SRA	QVQLQDSGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIWRSGVSLYYTDSVKGRFTISRDDAKMTVSLQMNSLKPEDTAVYYCAAEATFPTWSRGRFADYDYRGQGTQVTVSS

61	MP3D3BR	QVQLQESGGGLVQAGDSLRLSCTASGRSFSSYGMGWFRQAPGKDHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVSLQMNGLKPEDTAVYYCAAEATFPTWNRGTFADYDYRGQGTQVTVSS
62	MP3E5BR	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVSLQMNGLKPEDTAVYYCAAEATFPTWNRGSFADYDYRGQGTQVTVSS
63	MP3C7SRA	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIWRSGVSLYYADSVKGRFTISRDDAKMTVSLQMNSLKPEDTAVYYCAAEATFPTWNRGRFADYDYSGQGTQVTVSS
64	MP2F1BR	AVQLVESGGGLVQTGDSLRLSCVASGGTFSRYAMGWFRQAPGKEREFVARIGYSGRSISYATSVEGRFAISRDNAKNTVYLQMNSLKPEDTAVYYCASLVSGTLYQADYWGQGTQVTVSS
65	MP2C5BR	QVQLVESGGGLVQTGDSLRLSCVASGGTFSRYAMGWFRQPPGKERDFVARIGYSGQSISYATSVEGRFAISRDNAKNTVYLQMNSLKPEDTAVYYCASLVSGTLYKPNYWGQGTQVTVSS
66	MP2C10BR	QVKLEESGGGLVQAGGSLRLSCAASGLTYTVGWFRQAPGKEREFVAAISWSGGSALYADSVKGRFTISRDNAKNTVYLQMGSLEPEDTAYYSCAAPGTRYYGSNQVNYNYWGQGTQVTVSS
67	MP2G5SR	QVKLEESGGGLVQAGDSLRLSCAASGLTYTVGWFRQAPGKEREFVAAIDWSGGSALYADSVKGRFTISRDNTKNTVYLQMGSLEPEDTAVYWCAAPGTRYHGRNQVNYNYWGQGTQVTVSS
68	MP3B1SRA	QVQLQESGGGLVQPGGSLRLSCAASGFTSSNYAMSWVRQAPGKGLEWVSSINSRTGSITYADSVKGRFTITLDNAKNTLYLQMNSLKPEDTAVYYCASRVDDRVSRGQGTQVTVSS
69	MP2F10SR	QVQLVESGGGLVQAGGSLRLSCAASGRTISSFRMGWFRRAPGEEREFVAFVRSNGTSTYYADSVEGRFTITRDNAKNTVYLRMDSLKPEDTAVYYCAAATRDYGGSFDYWGQGTQVTVSS
70	MP3A7SRA	QVQLQDSGGGLVQAGGSLRLSCAASGRTFSSFRMGWFRRAPGEEREFVAFVRSNGTSTYYADSVEGRFTITRDNAKNTVYLRMDSLKPEDTAVYYCAAATRDYGGSFDYWGQGTQVIVSS
		Anti-mouse serum albumin VHH
71	MSA21	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEWVSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCTIGGSLNPGGQGTQVTVSS
72	MSAc16	AVQLVESGGGLVQAGDSLRLSCVVSGTTFSSAAMGWFRQAPGKEREFVGAIKWSGTSTYYTDSVKGRFTISRDNVKNTVYLQMNNLKPEDTGVYTCAADRDRYRDRMGPMTTTDFRFWGQGTQVTVSS
73	MSAc112	QVKLEESGGGLVQTGGSLRLSCAASGRTFSSFAMGWFRQAPGREREFVASIGSSGITTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTGLCYCAVNRYGIPYRSGTQYQNWGQGTQVTVSS
74	MSAc110	EVQLEESGGGLVQPGGSLRLSCAASGLTFNDYAMGWYRQAPGKERDMVATISIGGRTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCVAHRQTVVRGPYLLWGQGTQVTVSS
75	MSAc114	QVQLVESGGKLVQAGGSLRLSCAASGRTFSNYAMGWFRQAPGKEREFVAGSGRSNSYNYYSDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAASTNLWPRDRNLYAYWGQGTQVTVSS
76	MSAc116	EVQLVESGGGLVQAGDSLRLSCAASGRSLGIYRMGWFRQVPGKEREFVAAISWSGGTTRYLDSVKGRFTISRDSTKNAVYLQMNSLKPEDTAVYYCAVDSSGRLYWTLSTSYDYWGQGTQVTVSS

77	MSAcl19	QVQLVEFGGGLVQAGDSLRLSCAASGRSLGIYKMAWFRQVPGKEREFVAAISWSGGTTRYIDSVKGRFTLSRDNTKNMVYLQMNSLKPDDTAVYYCAVDSSGRLYWTLSTSYDYWGQGTQVTVSS
78	MSAcl5	EVQLVESGGGLVQAGGSLSLSCAASGRTFSPYTMGWFRQAPGKEREFLAGVTWSGSSTFYGDSVKGRFTASRDSAKNTVTLEMNSLNPEDTAVYYCAAAYGGGLYRDPRSYDYWGRGTQVTVSS
79	MScl11	AVQLVESGGGLVQAGGSLRLSCAASGFTLDAWPIAWFRQAPGKEREGVSCIRDGTTYYADSVKGRFTISSDNANNTVYLQTNSLKPEDTAVYYCAAPSGPATGSSHTFGIYWNLRDDYDNWGQGTQVTVSS
80	MSAcl15	EVQLVESGGGLVQAGGSLRLSCAASGFTFDHYTIGWFRQVPGKEREGVSCISSSDGSTYYADSVKGRFTISSDNAKNTVYLQMNTLEPDDTAVYYCAAGGLLLRVEELQASDYDYWGQGIQVTVSS
81	MSAcl8	AVQLVDSGGGLVQPGGSLRLSCTASGFTLDYYAIGWFRQAPGKEREGVACISNSDGSTYYGDSVKGRFTISRDNAKTTVYLQMNSLKPEDTAVYYCATADRHYSASHHPFADFAFNSWGQGTQVTVSS
82	MSAcl7	EVQLVESGGGLVQAGGSLRLSCAAYGLTFWRAAMAWFRRAPGKERELVVARNWGDGSTRYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAAVRTYGSATYDIWGQGTQVTVSS
83	MSAcl20	EVQLVESGGGLVQDGGSLRLSCIFSGRTFANYAMGWFRQAPGKEREFVAAINRNGGTTNYADALKGRFTISRDNTKNTAFLQMNSLKPDDTAVYYCAAREWPFSTIPSGWRYWGQGTQVTVSS
84	MSAcl4	DVQLVESGGGWVQPGGSLRLSCAASGPTASSHAIGWFRQAPGKEREFVVGINRGGVTRDYADSVKGRFAVSRDNVKNTVYLQMNRLKPEDSAIYICAARPEYSFTAMSKGDMDYWGKGTLVTVSS

Table 14: Sequence listing

Day of immunization	Llama 005 EGFr	Llama006 EGFr	Llama005 PDK1	Llama006 PDK1
0	100 µg	40 µg	40 µg	40 µg
7	100 µg		40 µg	
14	50 µg		20 µg	
21	50 µg	40 µg	20 µg	40 µg
28	50 µg		20 µg	
35	50 µg		20 µg	
42		20 µg		20 µg
70		20 µg		20 µg

Table 15: Immunisation scheme according to Example 32

llama	Source RNA	Elution conditions	Pfu EGFr	Pfu casein	Enrichment
005	Pool of the 3 libraries	0.2 M glycin, pH 2.4	1×10^7	1×10^4	1000
006	Pool of the 3 libraries	0.2 M glycin, pH 2.4	5×10^6	1×10^4	500

Table 16: Results of panning according to Example 35

llama	Source RNA	Elution conditions	Pfu PDK1	Pfu casein	Enrichment
005	Pool of the 3 libraries	0.2 M glycin, pH 2.4	1×10^8	1×10^4	10000
006	Pool of the 3 libraries	0.2 M glycin, pH 2.4	9×10^7	1×10^4	9000

Table 17: Results of panning according to Example 35

target	Llama005	Llama006
EGFr	26/95	38/95
PDK1	93/95	87/95

Table 18: Number of positive clones after screening according to Example 36

target	Llama005	Llama006
PDK1	56/93	63/87

Table 19: Number of inhibiting VHH vs number of VHH tested in inhibition ELISA according to Example 38.

SEQ	VHH#1A	VHH#7B	VHH#2B	VHH#3E	VHH#3G	VHH#10A	VHH#2G	VHH#1F	VHH#9C	VHH#11E	VHH#10C	VHH#4B	VHH#10D	VHH#12B	VHH#9E	VHH#3F
VHH#1A	1.000	0.601	0.764	0.596	0.622	0.600	0.682	0.629	0.609	0.601	0.614	0.818	0.642	0.747	0.596	0.604
VHH#7B	---	1.000	0.604	0.635	0.645	0.943	0.653	0.616	0.933	0.933	0.719	0.593	0.614	0.620	0.616	0.624
VHH#2B	---	---	1.000	0.620	0.645	0.611	0.682	0.661	0.629	0.620	0.637	0.796	0.634	0.951	0.620	0.645
VHH#3E	---	---	---	1.000	0.875	0.641	0.713	0.689	0.620	0.643	0.612	0.604	0.648	0.596	0.674	0.682
VHH#3G	---	---	---	---	1.000	0.651	0.779	0.740	0.637	0.637	0.653	0.645	0.689	0.622	0.708	0.716
VHH#10A	---	---	---	---	---	1.000	0.658	0.614	0.935	0.935	0.725	0.592	0.612	0.626	0.622	0.637
VHH#2G	---	---	---	---	---	---	1.000	0.741	0.653	0.669	0.685	0.666	0.746	0.650	0.701	0.717
VHH#1F	---	---	---	---	---	---	---	1.000	0.616	0.616	0.664	0.661	0.714	0.645	0.709	0.717
VHH#9C	---	---	---	---	---	---	---	---	1.000	0.941	0.743	0.601	0.622	0.645	0.600	0.616
VHH#11E	---	---	---	---	---	---	---	---	---	1.000	0.719	0.601	0.622	0.637	0.608	0.624
VHH#10C	---	---	---	---	---	---	---	---	---	---	1.000	0.650	0.606	0.637	0.600	0.632
VHH#4B	---	---	---	---	---	---	---	---	---	---	---	1.000	0.611	0.796	0.588	0.629
VHH#10D	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.619	0.674	0.674
VHH#12B	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.604	0.637
VHH#9E	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1.000	0.854
VHH#3F																1.000

Table 20: Fractional homologies between anti-TNF-alpha VHHs of the invention.

	% Homology																																		
	MP3D2SRA	MP3A3SR	MP3C5SR	MP3C1SR	MP3G8SR	P3D2BR	MP3H6SRA	MP3B4SRA	MP4E4BR	MP4H8SR	MP2F6SR	MP3D1BR	MP2B5BR	MP2C1BR	MP4A12SR	MP3F4SRA	MP3D3BR	MP3E5BR	MP3C7SRA	MP2F1BR	MP2C5BR	MP2C10BR	MP2G5SR	MP3B1SRA	MP2F10SR	MP3A7SRA	MP4C10SR	MP4D5BR	MP3F1SRA	MP6D6BR	MP6B1BR	MP6A8BR	MP6B12BR	MP6C11BR	MP6B10BR
MP3D2SRA	X	96	66	66	66	62	71	71	71	70	68	69	65	63	64	68	66	67	68	71	70	68	67	63	67	68	60	72	65	68	67	66	67	76	70
MP3A3SR	--	X	66	66	66	62	72	72	72	71	70	71	65	63	64	68	66	67	68	72	72	69	67	64	66	67	60	73	65	67	67	65	66	77	71
MP3C5SR	--	--	X	97	98	73	65	65	64	63	63	63	60	58	59	64	64	65	66	65	65	65	63	63	64	64	61	67	60	74	63	60	63	70	64
MP3C1SR	--	--	--	X	98	72	64	64	64	62	62	62	58	57	58	65	64	64	65	64	63	64	62	63	64	65	60	67	59	73	63	60	62	70	65
MP3G8SR	--	--	--	--	X	73	65	65	64	63	63	63	59	58	59	64	64	65	66	65	64	65	63	63	65	65	61	66	60	73	63	61	63	71	64
MP3D2BR	--	--	--	--	--	X	63	63	63	62	63	64	59	58	58	62	61	62	63	64	63	63	63	64	63	63	63	65	58	73	64	60	63	68	67
MP3H6SRA	--	--	--	--	--	--	X	100	97	97	80	81	67	68	67	75	71	73	75	73	71	73	71	66	75	75	63	71	69	71	71	68	70	82	70
MP3B4SRA	--	--	--	--	--	--	--	X	97	97	80	81	67	68	67	75	71	73	75	73	71	73	71	66	75	75	63	71	69	71	71	68	70	82	70
MP4E4BR	--	--	--	--	--	--	--	--	X	97	81	82	68	69	68	73	70	71	73	73	71	73	71	66	75	75	63	72	70	71	71	68	70	80	71
MP4H8SR	--	--	--	--	--	--	--	--	--	X	81	81	66	66	66	72	69	71	72	71	71	72	71	64	73	73	62	70	67	69	70	67	70	79	71
MP2F6SR	--	--	--	--	--	--	--	--	--	--	X	94	65	68	64	70	67	69	71	67	65	73	71	63	71	70	62	69	66	67	69	68	67	78	69
MP3D1BR	--	--	--	--	--	--	--	--	--	--	--	X	65	66	65	71	69	71	72	67	65	70	69	63	71	71	62	68	66	67	71	69	69	79	70
MP2B5BR	--	--	--	--	--	--	--	--	--	--	--	--	X	95	97	63	64	64	64	65	63	64	63	60	66	63	57	63	84	65	63	63	62	70	65
MP2C1BR	--	--	--	--	--	--	--	--	--	--	--	--	--	X	95	63	64	64	64	63	61	66	65	59	66	63	56	61	85	65	64	63	62	70	65
MP4A12SR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	63	64	64	64	62	60	63	62	59	65	63	56	61	84	64	63	63	62	70	65
MP3F4SRA	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	94	96	97	69	67	68	68	62	67	69	60	72	63	67	68	65	65	76	71
MP3D3BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	98	96	70	68	67	67	62	67	67	60	70	64	66	66	64	64	75	69
MP3E5BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	98	70	68	68	69	63	68	68	60	72	64	67	68	65	66	77	71
MP3C7SRA	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	71	69	69	70	63	69	69	61	72	64	68	68	66	66	78	71
MP2F1BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	94	66	67	63	68	67	61	70	64	68	65	64	64	74	67
MP2C5BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	66	67	63	67	65	62	69	63	67	64	62	63	73	67
MP2C10BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	94	62	68	66	59	67	66	69	68	64	68	74	73
MP2G5SR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	62	67	65	59	67	65	67	66	64	66	73	73
MP3B1SRA	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	66	65	91	67	60	67	69	68	69	69	65
MP2F10SR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	97	61	67	65	71	66	65	67	77	68
MP3A7SRA	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	61	68	63	71	65	65	67	77	69
MP4C10SR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	64	58	65	64	63	66	66	63
MP4D5BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	64	69	68	65	67	76	73
MP3F1SRA	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	65	64	64	63	71	68
MP6D6BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	70	65	70	77	73
MP6B1BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	78	81	76	71
MP6A8BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	75	74	66
MP6B12BR	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	X	73	68
MP6C11BR																																		X	77
MP6B10BR																																			X

Table 21: Percentage homologies between anti-IFN-gamma VHHs of the invention

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Parallel expression of macrophage metalloelastase (MMP-12) in duodenal and skin lesions of patients with dermatitis herpetiformis.

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Oncology 2002;63(4):378-84.
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J Invest Dermatol 2000 Jun;114(6):1113-9
Expression of human macrophage metalloelastase (MMP-12) by tumor cells in skin cancer.

CLAIMS

1. A polypeptide construct comprising at least one single domain antibody directed against IgE.

2. A polypeptide construct according to claim 1 wherein at least one single domain antibody is a *Camelidae* VHH.

3. A polypeptide construct according to claims 1 and 2 wherein at least one single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 11.

4. A polypeptide construct according to any of claims 1 to 3, wherein the number of anti-IgE single domain antibodies is at least two.

5. A polypeptide construct according any of claims 1 to 4, wherein at least one single domain antibody is a humanized *Camelidae* VHH.

6. A polypeptide construct according to any of claims 1 to 5, wherein a single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

7. A polypeptide construct according to any of claims 1 to 6, wherein the polypeptide construct is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length polypeptide construct.

8. A nucleic acid encoding a polypeptide construct according to any of claims 1 to 7.

9. A polypeptide construct according to any of claims 1 to 7 for treating and/or preventing and/or alleviating disorders relating to inflammatory processes.

10. Use of a polypeptide construct according to any of claims 1 to 7 for the preparation of a medicament for treating and/or preventing and/or alleviating disorders relating to inflammatory reactions.

11. A method for delivering an anti-target compound to a subject for the treatment of a disorder without being inactivated by administering thereto a polypeptide construct comprising one or more single domain antibodies directed against said target.

12. A method according to claim 11 wherein said target is located in the gut system, and said a polypeptide construct is delivered orally.

13. A method according to claim 11 wherein said target is located in vaginal and/or rectal tract, and said a polypeptide construct is delivered to the vaginal and/or rectal tract.

14. A method according to claim 11 wherein said target is located in nose, upper respiratory tract and/or lung, and said a polypeptide construct is delivered to nose, upper respiratory tract and/or lung.

15. A method according to claim 11 wherein said target is located in intestinal mucosa, and said a polypeptide construct is delivered orally.

16. A method according to claim 11 wherein said target is located in the tissues beneath the tongue, and said a polypeptide construct is delivered to the tissues beneath the tongue.

17. A method according to claim 11 wherein said target is located in the skin, and said a polypeptide construct is delivered topically.

18. A method according to claim 11 wherein said target is in, or accessible via the blood, and said a polypeptide construct is delivered orally, to the vaginal and/or rectal tract, nasally, by inhalation though the mouth or nose, to the tissues beneath the tongue, or topically.

19. A polypeptide construct comprising at least one single domain antibody directed against a target, for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the gastric environment without being inactivated.

20. A polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of the intestinal mucosa without being inactivated

21. A polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of the nose, upper respiratory tract and/or lung without being inactivated

22. A polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by an anti-target therapeutic compound that is able pass through the wall of virginal and/or rectal tract without being inactivated

23. A polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by a therapeutic compound that is able pass through the tissues beneath the tongue without being inactivated

24. A polypeptide construct comprising at least one single domain antibody directed against a target for use in treating, preventing and/or alleviating the symptoms of disorders which are susceptible to modulation by a therapeutic compound that is able pass through the skin without being inactivated

25. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is TNF-alpha and the disorder is inflammation.

26. A method or polypeptide according to claim 25, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 12 to 14.

27. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is CEA and the disorder colon cancer.

28. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is EGFR and the disorder is any of head, neck, lung and colon cancer.

29. A method or polypeptide construct according to claim 28, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 23 to 44

30. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of *Helicobacter pylori* and the disorder is any of indigestion, gastritis.

31. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of *Mycobacterium tuberculosis* and the disorder is tuberculosis.

32. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of *influenza* virus and the disorder is flu.

33. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of IgE and the disorder is allergic response.

34. A method or polypeptide construct according to claim 33, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 1 to 11

35. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of MMP and the disorder is cancer.

36. A method or polypeptide construct according to claim 35, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 15 to 22

37. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24, wherein said target is antigen of IFN-gamma and the disorder is any of cancer, transplant rejection, auto immune disorder.

38. A method or polypeptide construct according to claim 37, wherein a single domain antibody corresponds to a sequence represented by any of SEQ ID NOs: 45 to 70

39. A method according to any of claims 11 to 18 or polypeptide construct according to any of claim 19 to 24 wherein said target is any of antigen of *Helicobacter pylori,* antigen of *Mycobacterium tuberculosis*, antigen of *influenza* virus.

40. A polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, and at least one single domain antibody directed against a therapeutic target.

41. A polypeptide construct comprising at least one single domain antibody directed against an internalising cellular receptor, and at least one therapeutic polypeptide or agent.

42. A polypeptide construct according to claims 40 and 41 wherein said internalising cellular receptor is Epidermal Growth Factor receptor.

43. A polypeptide construct according to claim 42 wherein a single domain antibody directed against an internalising cellular receptor corresponds to a sequence represented by SEQ ID NO: 23 to 44.

44. A polypeptide construct according to claims 40 and 41 wherein said internalising cellular receptor is any of LDL receptor, FGF2r, ErbB2r, transferring receptor, PDGr, VEGr, or PsmAr.

45. A polypeptide construct according to any of claims 40 to 44 wherein a single domain antibody directed against a therapeutic target, is directed against PDK1.

46. A polypeptide construct according to claim 45 for use in treating cancer

47. A polypeptide construct according to any of claims 40 to 44 wherein a single domain antibody directed against a therapeutic target is directed against any of GSK1, Bad, caspase and Forkhead.

48. A polypeptide construct according to claim 47 for use in treating cancer.

49. Method for delivering an anti-target therapeutic compound to the interior of a cell comprising administering to a subject a polypeptide construct according to any of claims 40 to 48.

50. Method for delivering an anti-target therapeutic compound to the interior of a cell without being inactivated comprising administering to a subject a polypeptide construct according to any of claims 40 to 49.

51. A method according to claim 50 wherein said cell is located in the gut system, and said a polypeptide construct is delivered orally.

52. A method according to claim 50 wherein said cell is located in vaginal and/or rectal tract, and said a polypeptide construct is delivered to the vaginal and/or rectal tract.

53. A method according to claim 50 wherein said cell is located in nose, upper respiratory tract and/or lung, and said a polypeptide construct is delivered to nose, upper respiratory tract and/or lung.

54. A method according to claim 50 wherein said cell is located in intestinal mucosa, and said a polypeptide construct is delivered orally.

55. A method according to claim 50 wherein said cell is located in the tissues beneath the tongue, and said a polypeptide construct is delivered to the tissues beneath the tongue.

56. A method according to claim 50 wherein said cell is located in the skin, and said a polypeptide construct is delivered topically.

57. A method according to claim 50 wherein said cell is in, or accessible via the blood, and said a polypeptide construct is delivered orally, to the vaginal and/or rectal tract, nasally, by inhalation though the mouth or nose, to the tissues beneath the tongue, or topically.

58. A polypeptide construct according to any of claims 1 to 7, 19 to 24, 40 to 48, or a method according to any of claims 11 to 18, 25 to 39, 49 to 57, wherein the single domain antibodies are humanized *Camelidae* VHHs.

59. A polypeptide construct according to any of claims 1 to 7, 19 to 24, 40 to 48, 58, or a method according to any of claims 11 to 18, 25 to 39, 49 to 57, wherein said single domain antibody is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length single domain antibody.

60. A polypeptide construct according any of claims 1 to 7, 19 to 24, 40 to 48, 58 and 59 or a method according to any of claims 11 to 18, 25 to 39, 49 to 59, wherein the polypeptide construct is an homologous sequence, a functional portion, or a functional portion of an homologous sequence of the full length polypeptide construct.

61. A polypeptide construct according to any of claims 1 to 7, 19 to 24, 40 to 48, 58 to 60 or a method according to any of claims 11 to 18, 25 to 39, 49 to 60 wherein said single domain antibodies are *Camelidae* VHHs.

62. A nucleic acid encoding a polypeptide construct according to any of claims 1 to 7, 19 to 24, 40 to 48, 58 to 61.

63. A composition comprising a polypeptide construct as defined in any of the preceding claims, together with a pharmaceutical carrier.

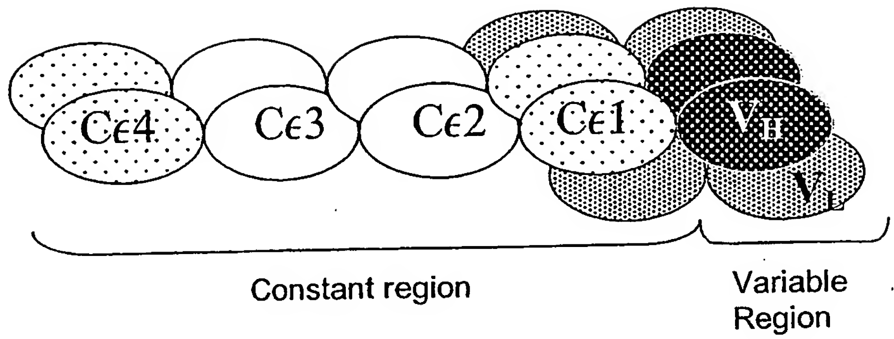


Figure 1

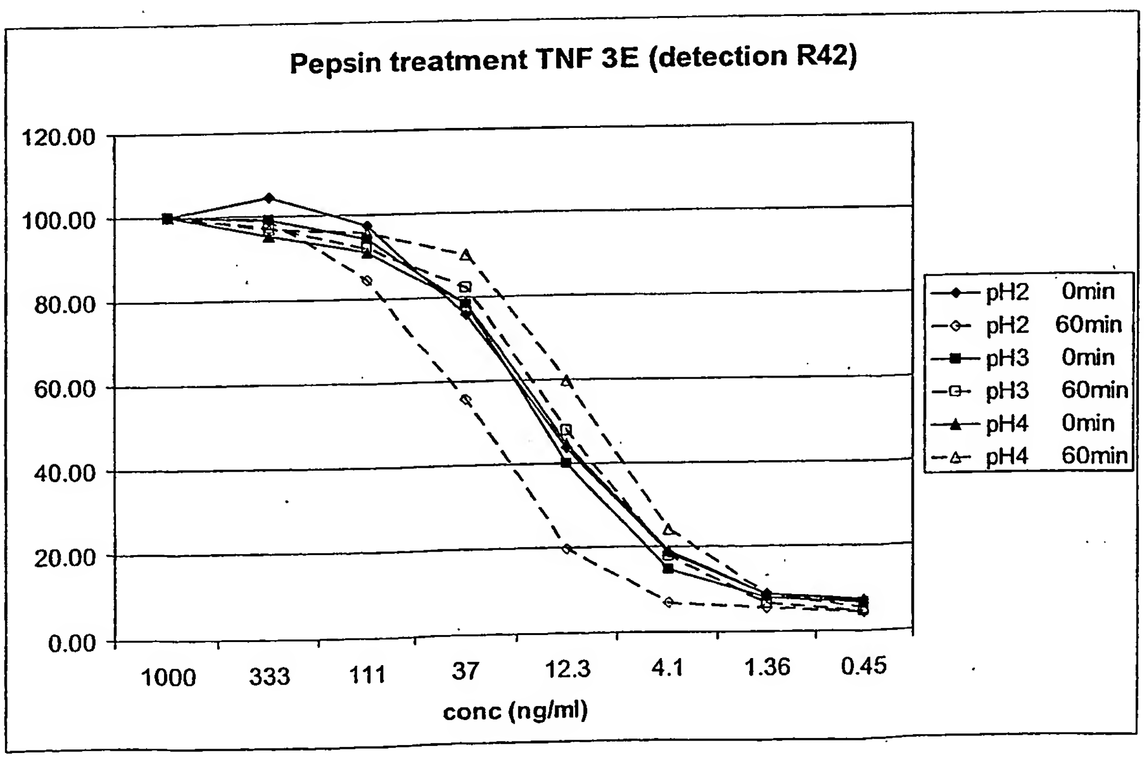


Figure 2

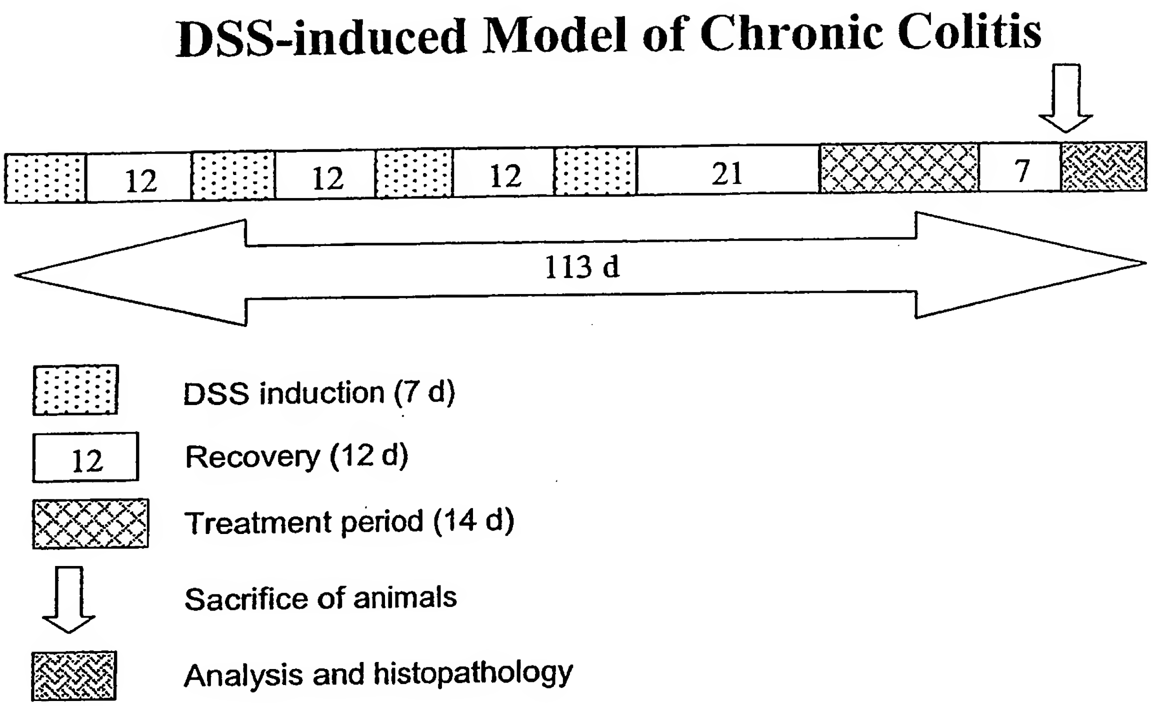

DSS-induced Model of Chronic Colitis
12
12
12
21
7
113 d
DSS induction (7 d)
12
Recovery (12 d)
Treatment period (14 d)
Sacrifice of animals
Analysis and histopathology

Figure 3

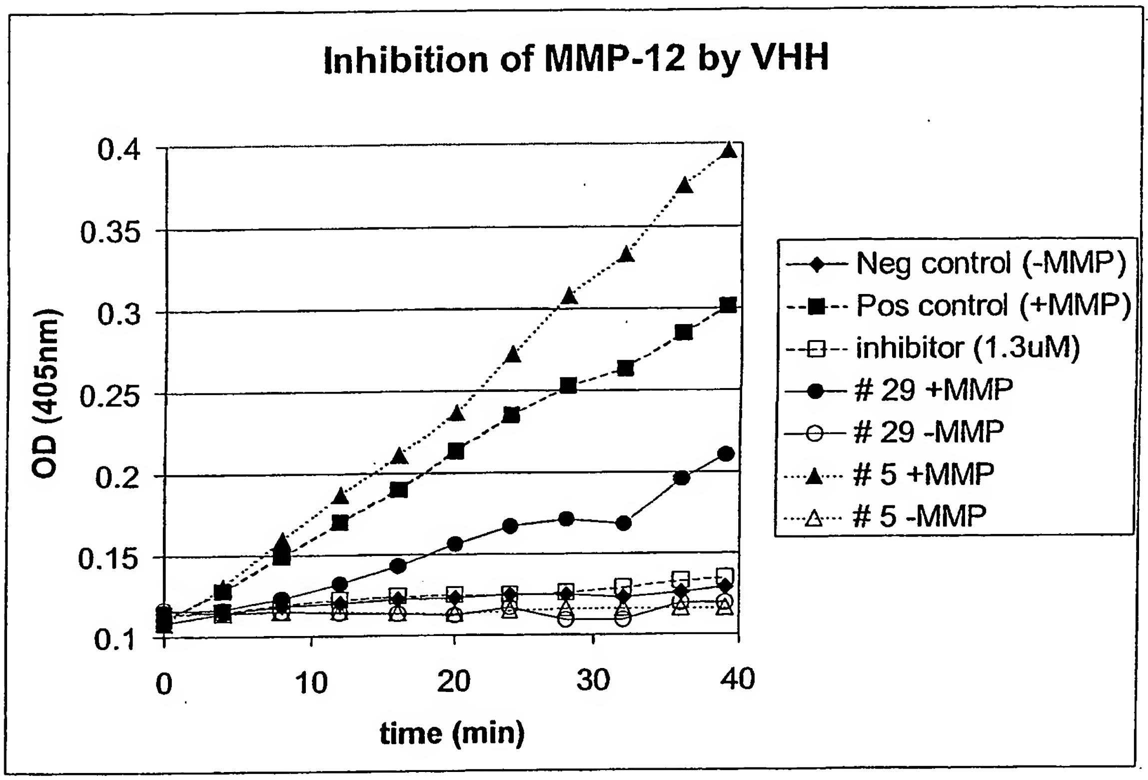

Inhibition of MMP-12 by VHH
0.4
0.35
0.3
0.25
0.2
0.15
0.1
OD (405nm)
0
10
20
30
40
time (min)
Neg control (-MMP)
Pos control (+MMP)
inhibitor (1.3uM)
29 +MMP
29 -MMP
5 +MMP
5 -MMP

Figure 4

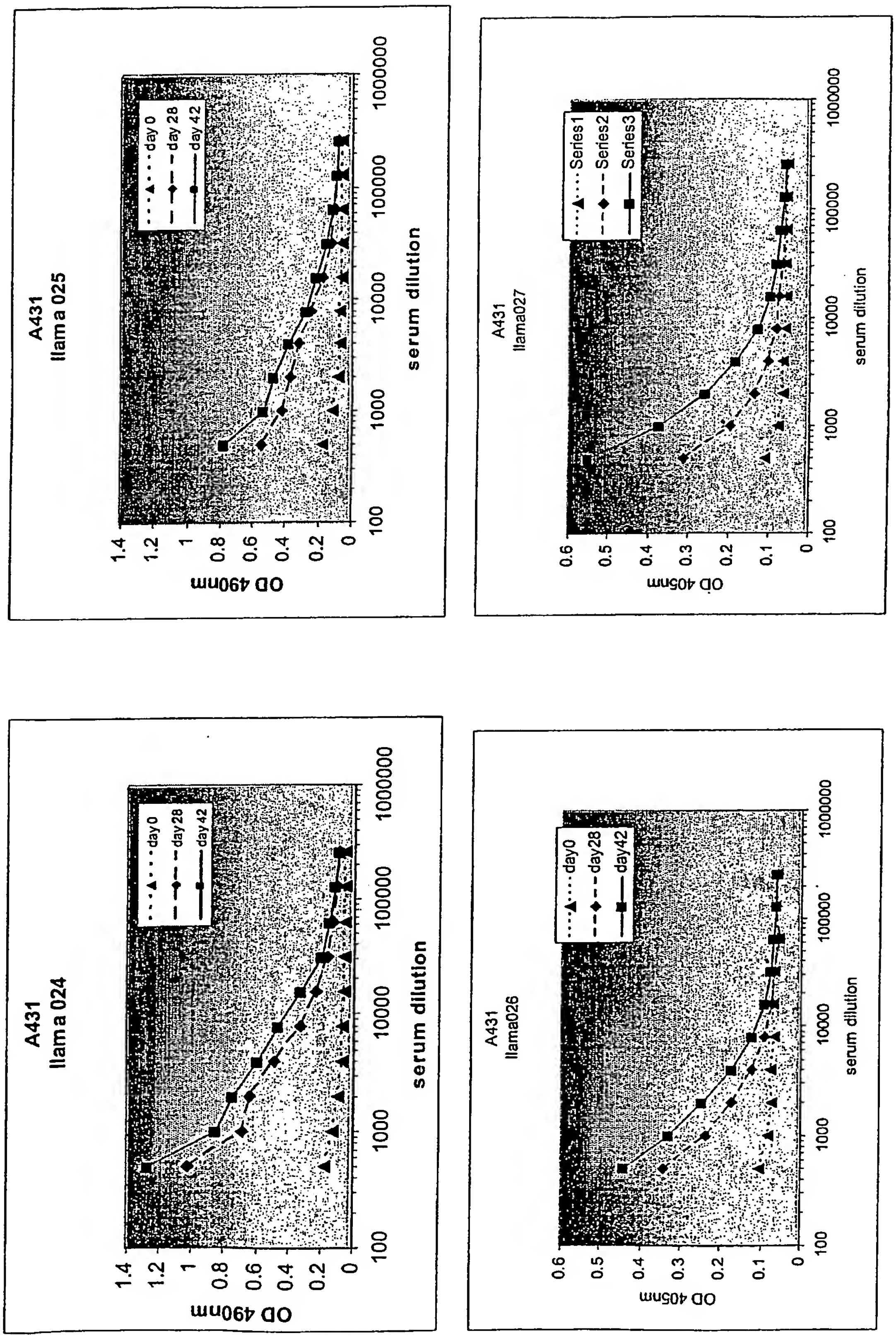

A431
llama 025
OD 490nm
serum dilution
day 0
day 28
day 42
A431
llama027
OD 405nm
serum dilution
Series1
Series2
Series3
A431
llama 024
OD 490nm
serum dilution
day 0
day 28
day 42
A431
llama026
OD 405nm
serum dilution
day0
day28
day42

Figure 5

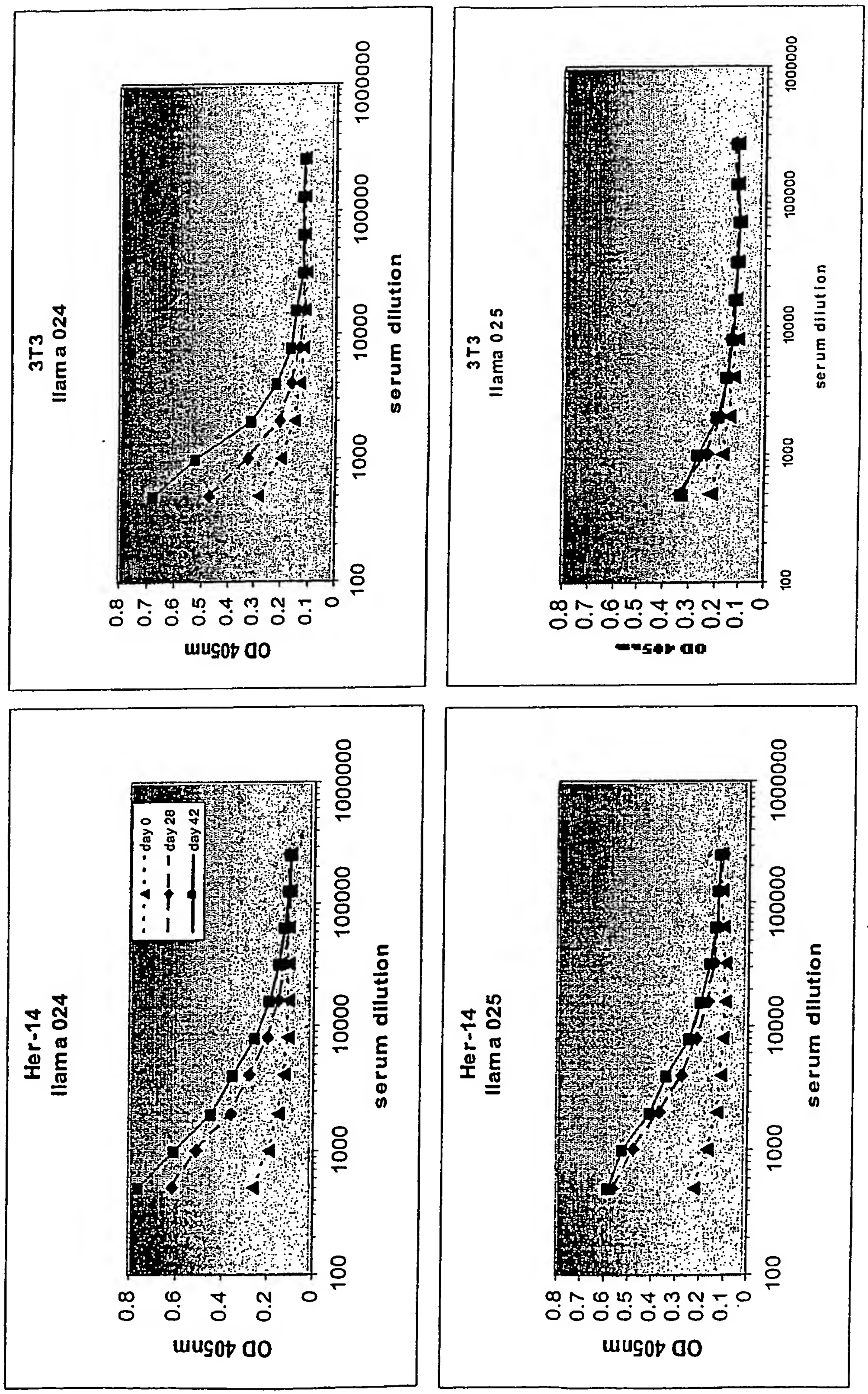

3T3
llama 024
Her-14
llama 024
3T3
llama 025
Her-14
llama 025
OD 405nm
serum dilution
day 0
day 28
day 42
100
1000
10000
100000
1000000

Figure 6 - 1

6/14

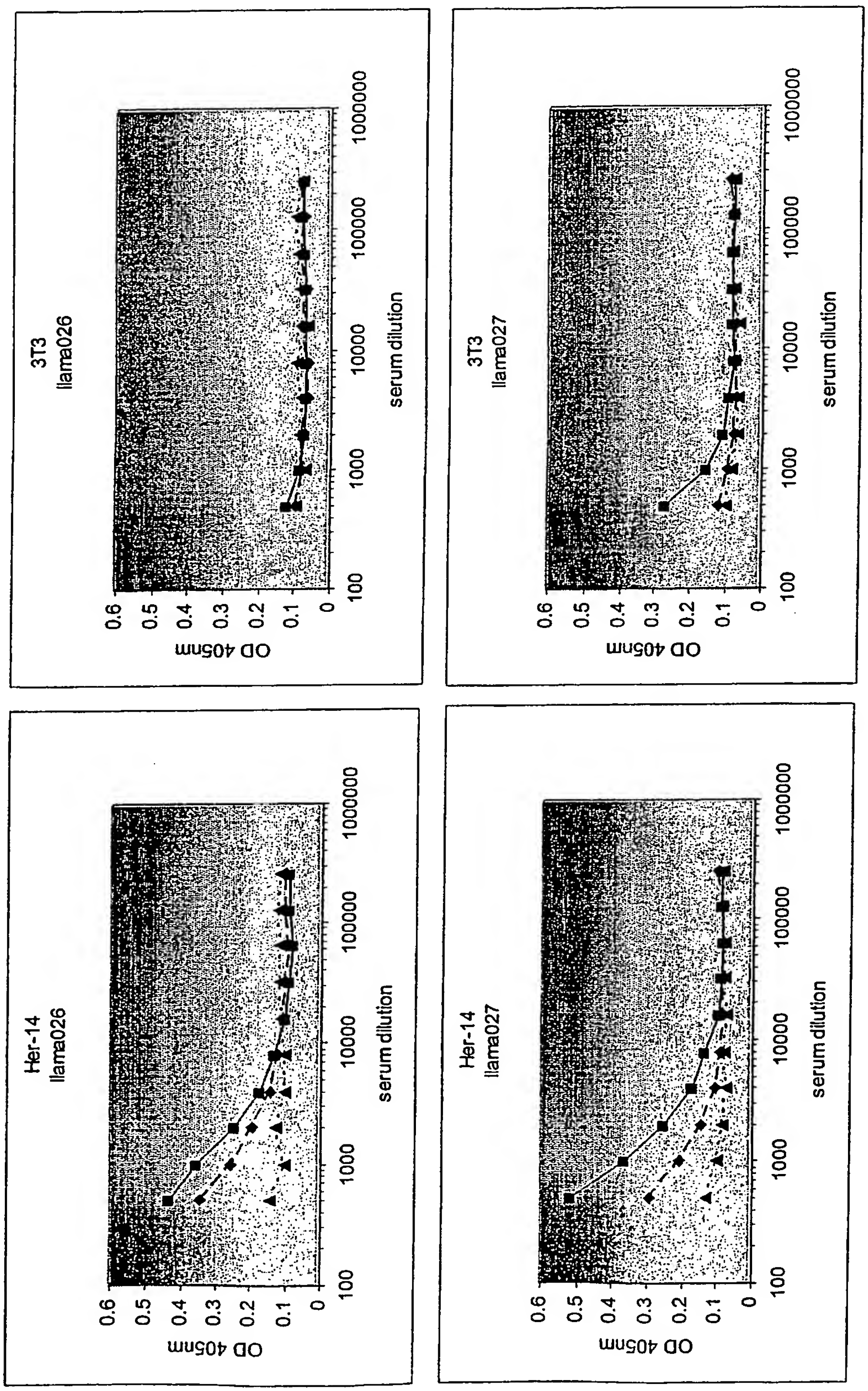
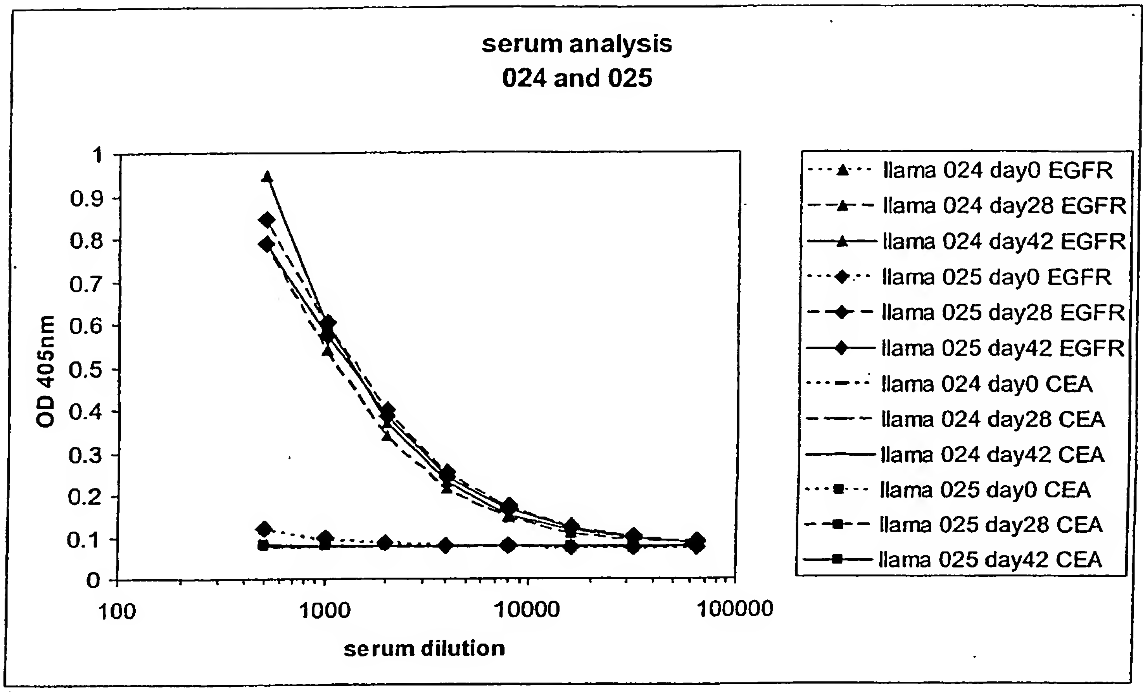


Figure 6- 2


serum analysis
024 and 025
OD 405nm
serum dilution
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
1000
10000
100000
llama 024 day0 EGFR
llama 024 day28 EGFR
llama 024 day42 EGFR
llama 025 day0 EGFR
llama 025 day28 EGFR
llama 025 day42 EGFR
llama 024 day0 CEA
llama 024 day28 CEA
llama 024 day42 CEA
llama 025 day0 CEA
llama 025 day28 CEA
llama 025 day42 CEA

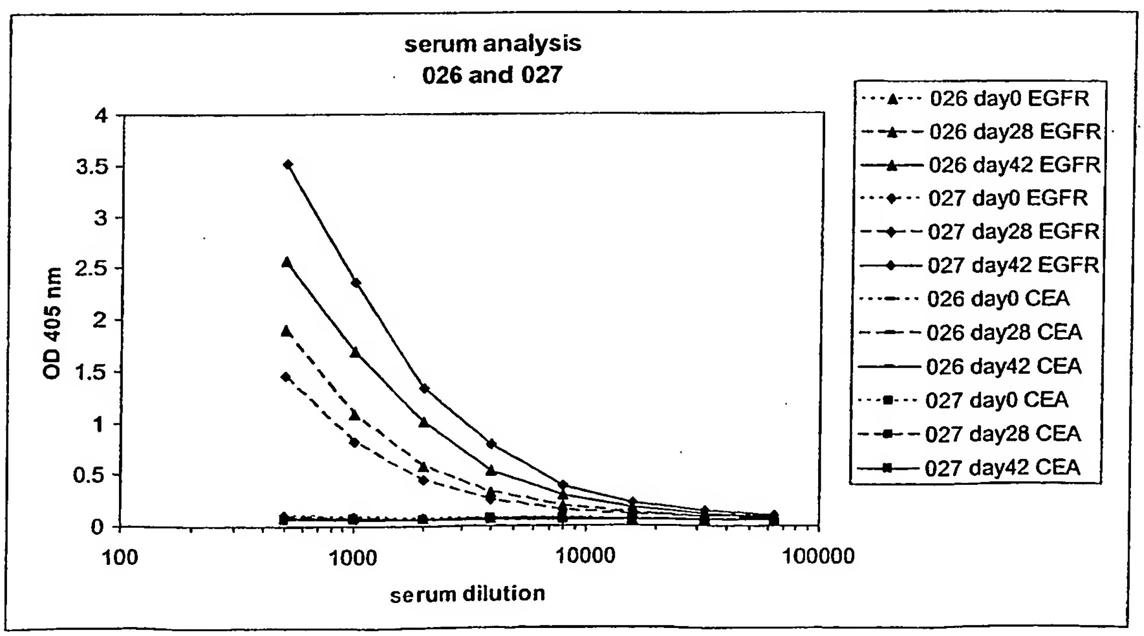

serum analysis
026 and 027
OD 405 nm
serum dilution
4
3.5
3
2.5
2
1.5
1
0.5
0
100
1000
10000
100000
026 day0 EGFR
026 day28 EGFR
026 day42 EGFR
027 day0 EGFR
027 day28 EGFR
027 day42 EGFR
026 day0 CEA
026 day28 CEA
026 day42 CEA
027 day0 CEA
027 day28 CEA
027 day42 CEA

Figure 7

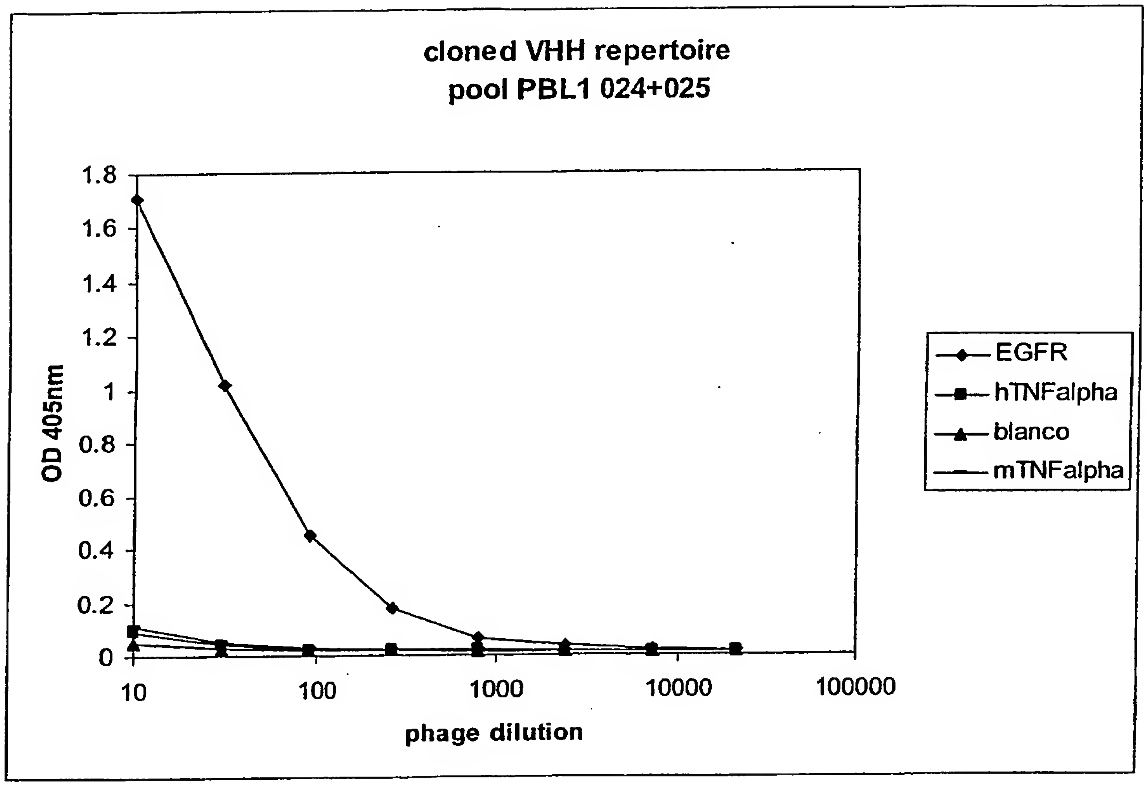

cloned VHH repertoire
pool PBL1 024+025
OD 405nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10
100
1000
10000
100000
phage dilution
EGFR
hTNFalpha
blanco
mTNFalpha

Figure 8

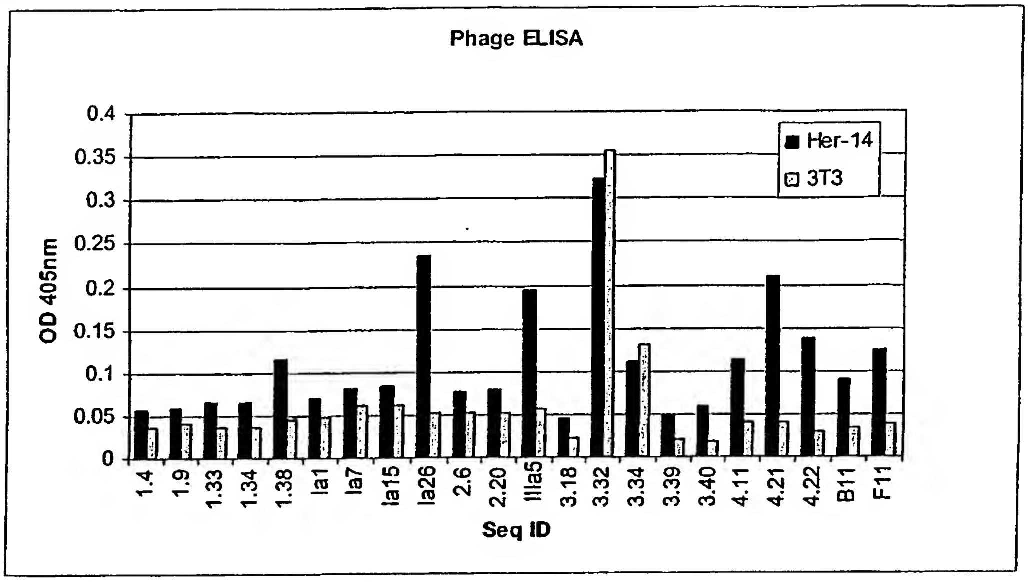
	< FR1 >	CDR1	< FR2 >	CDR2
1.33	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SHYMS	WFRQAPGKEREFVA	AITSSSRTYYTESVKG
3.14	AVQLVESGGGLVQPGGSLRLSCAASGFTFS	SHYMS	WFRQAPGKEREFVA	AITSSSRTYYTESVKG
2.34	QVKLEESGGGLVQPGDSLRLSCAASGFTFS	SHYMS	WFRQAPGKEREFVA	AITSSSRTYYTESVKG
1.4	EVQLVESGGGLVQAGGSLRLSCAASGRTFS	NYVMG	WFRQAPGKERDFVV	GIGRSGGDNTYYADSVKG
Ia9	QVQLVESGGGLVQAGGSLRLSCAASGRTFS	NYVMG	WFRQAPGKERDFVV	GIGRSGGDNTYYADSVKG
2.20	EVQLVESGGGLVQAGGSLRLSCAASGRSFS	SYAMA	WFRQAPGKEREFVA	AISWGGGSTYYAVSVKG
IIIa6	EVQLVESGGGLVQAGGSLRLSCAASGRSFS	SYAMA	WFRQAPGKEREFVA	AISWGGGSTYYAVSVKG
Ia26	QVQLQESGGGSVQAGGSLKLSCAASGRGFS	RYAMG	WFRQAPGQDREFVA	TISWTNSTDYADSVKG
IIIa42	EVQLVESGGGSVQAGGSLKLSCAASGRSFS	TYAMG	WFRQAPGQDREFVA	TISWTDSTDYADSVKG
Ia33	QVQLQESGGGSVQAGGSLKLSCTASGRRFS	TYAVG	WFRQAPGQDREFVA	TISWTNSTDYADSVKG
3.1	EVQLVESGGGSVQAGGSLKLSCTASGRRFS	TYAVG	WFRQAPGQDREFVA	TISWTNSTDYADSVKG
Ia1	QVQLQESGGGLVQAGGSLLLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AINWSGGSTSYADSVKG
Ia21	QVQLQESGGGLVQAGGSLLLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AINWSGGSTSYADSVKG
IIIa3	QVQLQESGGGLVQAGGSLLLSCAASGRTFS	SYAMG	WFRQAPGKEREFVA	AINWSGGSTSYADSVKG
1.9	QVQLQESGGGLVKAGGSLRLSCAASGRTFS	SYVMG	WFRQAPGKEREFVG	AIHWSGGRTYYADSVKG
1.34	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	KYAMG	WFRQAPGKEREFVS	AISWSDGSTYYADSVKG
Ia10	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	KYAMG	WFRQAPGKEREFVS	AISWSDGSTYYADSVKG
2.6	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	NYAMG	WFRQAPGKEREFVA	AINWGGGNTYYADSVKG
3.34	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	SYAIG	WFRQAPGKEREFVA	AISWGGSNTYYADSVKG
1.38	QVQLQDSGGGLVQAGDSLRLSCAASGRSFG	GYAMG	WFRQAPGKEREFVA	AISWSGGSTYYADSLKG
3.32	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	GYAMG	WFRQAPGEEREFVA	AISWRGTSTYYGDSAKG
4.43	QVQLQESGGGLVQAGGSLRLSCAASGRTFS	GYAMG	WFRQAPGEEREFVA	AISWRGTSTYYGDSAKG
Ia15	QVQLQDSGGGLVQAGGSLRLSCAASGGTFS	SYAMG	WFRQAPGKEREFVA	AIGLNTYYADSVKG
Ia7	QVQLQESGGRLVQTGGSLRLSCAASGGTFG	TYALG	WFRQAPGKEREFVA	AISRFGSTYYADSVKG
3.39	QVQLQESGGGLVQTGGSLRLSCAASGRYIM	G	WFRQAPGKEREFVA	GISRSGASTAYADSVKD
3.40	QVKLEESGGGLVQAGGSLRLSCSASGLTFS	NYAMA	WFRQAPGKEREFVA	TISQRGGMRHYLDSVKD
4.22	QVKLEESGGGLVQAGGSLRLSCAASGSIFS	INAMG	WYRQAPGKQRELVA	RITGTGTGITGAVSTNYADSVKG
4.11	QVKLEESGGGLVQAGDSLRLSCAASGRSFS	SITMG	WFRQAPGKERQFVS	AINSNGNRYYADSVKG
4.21	EVQLVESGGGLVQAGGSLRLSCAVSGRTFS	SMG	WFRQAPGKEREFVA	TINLSGDRTDYADSVKG
IIIa5	EVQLVESGGGLVQAGGSLRLSCTASGRTFS	SYAMG	WFRQTPGKEREFVA	AITSSGGSTYYADSVKG
3.18	EVQLVESGGGLVQPGGSLRLSCVASGFTFA	DYAMS	WVRQAPGKGLQWVS	SISYNGDTTYYAESMKD

Figure 9 - 1

	< FR3 >	.CDR3	< FR4 >
1.33	RFTISRDNAKNTVYLQMNSLKSEDTAVYYCAA	DRTFYGSTWSKYDY	RGQGTQVTVSS
3.14	RFTISRDNAKNTVYLQMNSLKSEDTAVYYCAA	DRTFYGSTWSKYDY	RGQGTQVTVSS
2.34	RFTISRDNAKNTVYLQMDSLKSEDTAVYYCAA	DRTFYGSTWSKYDY	RGQGTQVTVSS
1.4	RFTISWDNAKNTMYLQMNSLKPEDTAVYYCAA	STYSRDTIFTKWANYNY	WGQGTQVTVSS
Ia9	RFTISWDNAKNTMYLQMNSLKPEDTAVYYCAA	STYSRDTIFTKWANYNY	WGQGTQVTVSS
2.20	RFTISRDNAKNTVYLQMNSLKPEDTARYYCAA	DETFHSSAYGEYEY	WGQGTQVTVSS
IIIa6	RFTISRDNAKNTVYLQMNSLKPEDTARYYCAA	DETFHSSAYGEYEY	WGQGTQVTVSS
Ia26	RFAISRDNAKNTAYLQMNSLKPEDTAVYYCAA	DKWASSTRSIDYDY	WGQGIQVTVSS
IIIa42	RFTISRDNAKNTGYLQMNSLKPEDTAVYYCAA	DRWASSRRNVDYDY	WGQGTQVTVSS
Ia33	RFTISRDNAKNTGYLQMNSLKPEDTSVYVCAA	DKWSSSRRSVDYDY	WGQGTQVTVSS
3.1	RFTISRDNAKNTGYLQMNSLKPEDTSVYVCAA	DKWSSSRRSVDYDY	WGQGTQVTVSS
Ia1	RFTISRDNTKNTVYLQMNSLKPEDTAAFYCAA	TYNPYSRDHYFPRMTTEYDY	WGQGTQVTVSS
Ia21	RFTISKDNTKNTVYLQMNSLKPEDTAAFYCAA	TYNPYSRDHYFPRMTTEYDY	WGQGTQVTVSS
IIIa3	RFTISRDNTKNTVYLQMNSLKPEDTAAFYCAA	TYNPYSRDHYFPRMTTEYDY	WGQGTQVTVSS
1.9	RFTISSDNAKNTLYLQMNSLKPEDTAVYYCAA	SRIIYSYVNYVNPGEYDY	WGQGTQVTVSS
1.34	RFTISRDNAKNTVYLQVNSLKPEDTAVYYCAA	TYLVDVWAVHVPIRPYEYDY	WGQGTQVTVSS
Ia10	RFTISRDNAKNTVYLQVNSLKPEDTAVYYCAA	TYLVDVWAVHVPIRPYEYDY	WGQGTQVSVSS
2.6	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA	SEWGGSDYDHDYDY	WGQGTQVTVSS
3.34	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA	GEVSNSDYAYEYDY	WGQGTQVTVSS
1.38	RFTISRDNAKNTVYLQMNSLKPEDTALYYCAA	GLRPSPNYNHER-SYDY	WGQGTQVTVSS
3.32	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA	GSHSDYAPDYDY	WGQGTQVTVSS
4.43	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA	GSHSDYAPDYDY	WGQGTQVTVSS
Ia15	RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA	RTSGVVGGTPKRYDY	WGQGTQVTVSS
Ia7	RFTISRDNANNTVYLEMNSLKPEDTAVYYCAA	REGVALGLRNDANY	WGQGTQVTVSS
3.39	RFTISRDSALNTVYLQMNSLKAEDTAVYFCAA	ALAIRLGIPRGETEYEY	WGQGTQVTVSS
3.40	RFTISRDNAKNTVYLQMNSLKPDDTAVYYCAA	DLMYGVDRRYDY	WGRGTQVTVSS

Figure 9 - 2

A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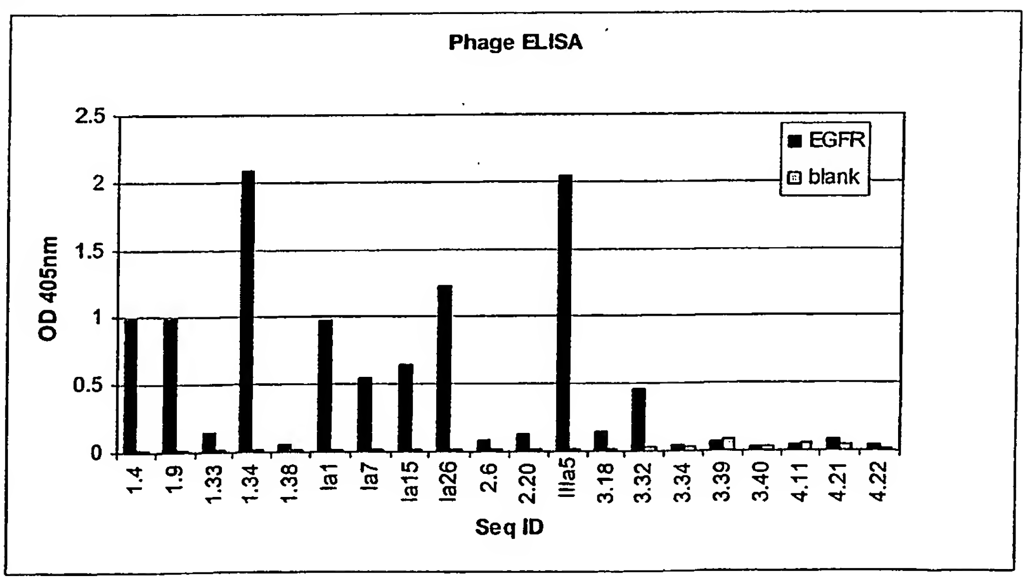
0.3
0.25
0.2
0.15
0.1
0.05
0
Her-14
3T3
1.4
1.9
1.33
1.34
1.38
Ia1
Ia7
Ia15
Ia26
2.6
2.20
IIIa5
3.18
3.32
3.34
3.39
3.40
4.11
4.21
4.22
B11
F11
Seq ID

B


Phage ELISA
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2
1.5
1
0.5
0
EGFR
blank
1.4
1.9
1.33
1.34
1.38
Ia1
Ia7
Ia15
Ia26
2.6
2.20
IIIa5
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3.40
4.11
4.21
4.22
Seq ID

Figure 10

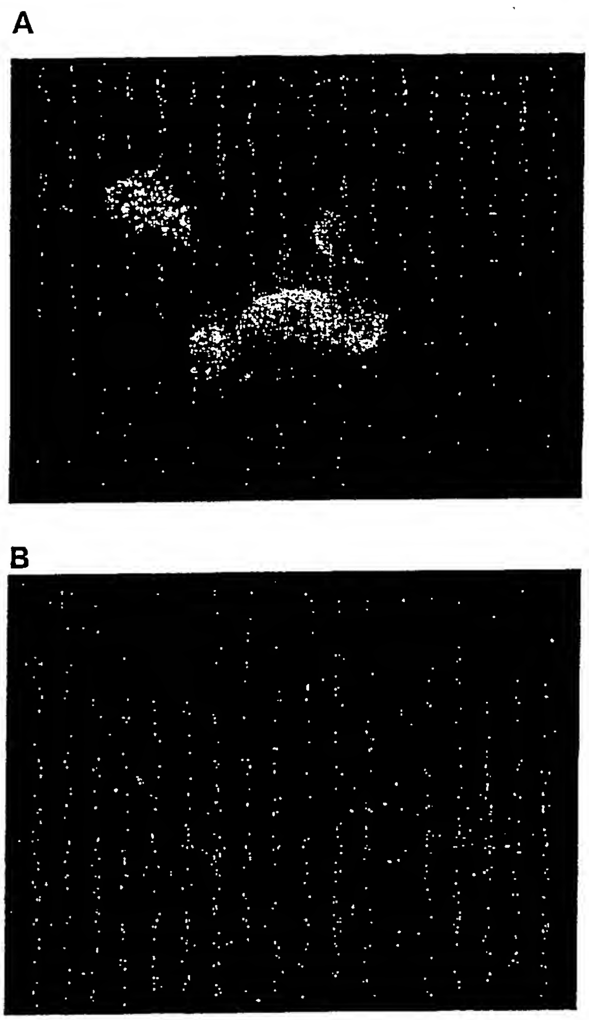
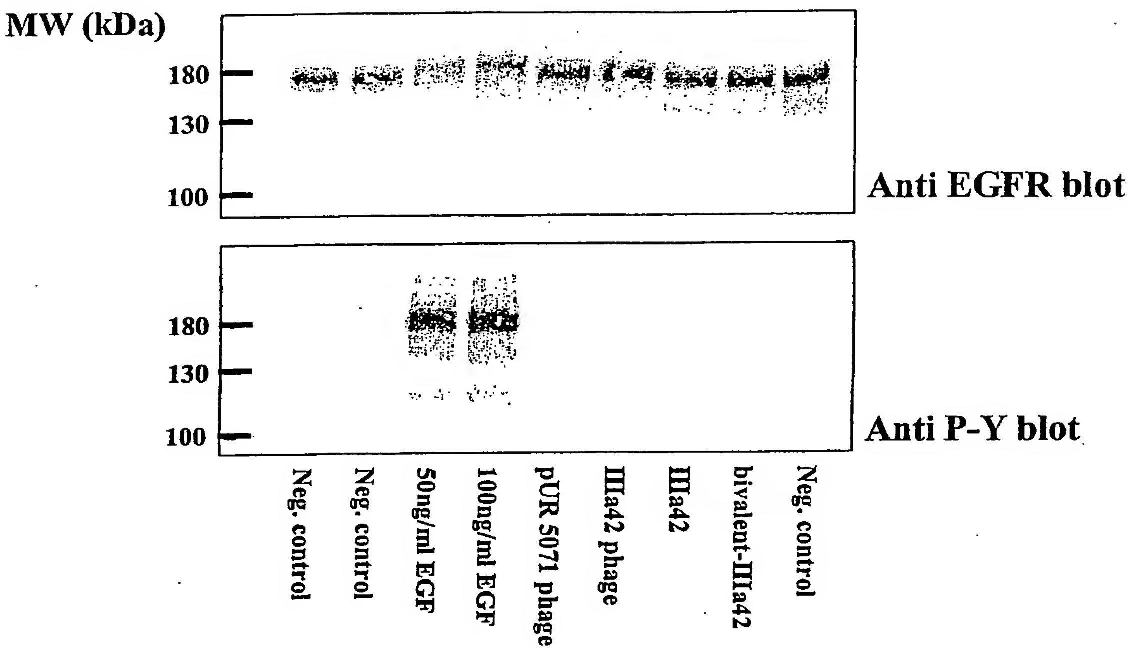


Figure 11 - 1

C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130
100
Anti EGFR blot
180
130
100
Anti P-Y blot
Neg. control
Neg. control
50ng/ml EGF
100ng/ml EGF
pUR 5071 phage
IIIa42 phage
IIIa42
bivalent-IIIa42
Neg. control

Figure 11 – 2

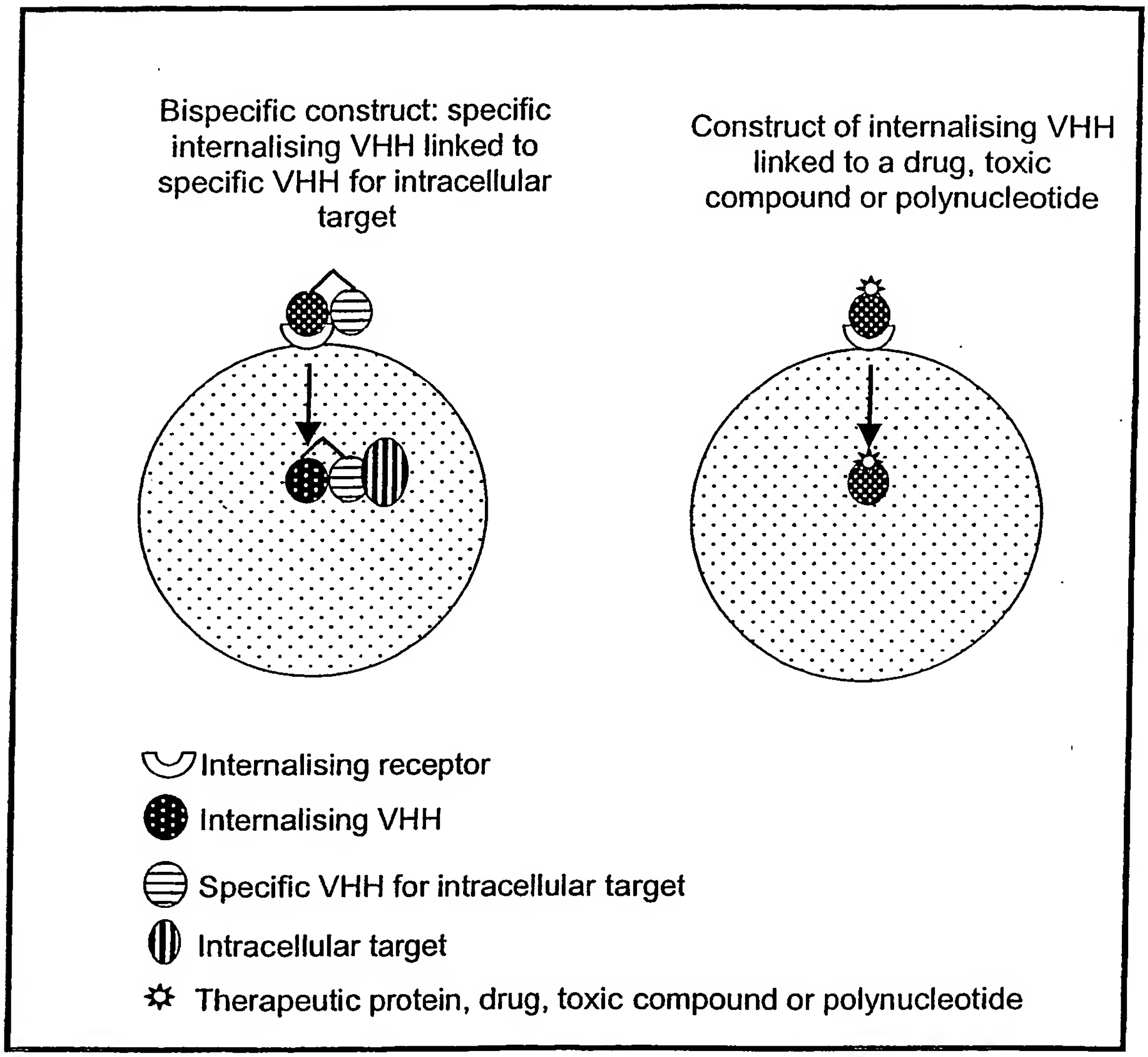
Bispecific construct: specific internalising VHH linked to specific VHH for intracellular target
Construct of internalising VHH linked to a drug, toxic compound or polynucleotide
Internalising receptor
Internalising VHH
Specific VHH for intracellular target
Intracellular target
Therapeutic protein, drug, toxic compound or polynucleotide

Figure 12